TARGETING MCT4 FOR TREATMENT OF ADVANCED PROSTATE CANCERS: INHIBITING CELL PROLIFERATION AND ENHANCING ANTICANCER IMMUNITY THROUGH SUPPRESSING LACTIC ACID SECRETION AND ELEVATED GLYCOLYSIS

by

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Abstract

Prostate cancer (PCa) is the most commonly diagnosed non-cutaneous cancer in North American males and a leading cause of cancer deaths. The lack of effective treatment options for advanced PCa such as AR-positive castration-resistant PCa (CRPC-AD) and the highly aggressive AR-negative CRPC, e.g. neuroendocrine PCa (CRPC-NE) presents a critical, unmet need for the development of novel therapeutics. Altered metabolism in the form of elevated aerobic glycolysis is a common cancer characteristic. Here we propose a novel conceptual understanding for the central, functional role of excessive cancer-generated lactic acid. In particular, the acidification of the tumor microenvironment via increased MCT4-mediated lactic acid secretion can facilitate multiple crucial cancer-promoting processes, including proliferation, tissue invasion/metastasis, angiogenesis, and suppression of local anticancer immunity. As such, the inhibition of MCT4 could be an effective therapeutic strategy broadly impacting multiple downstream lactate-associated tumour-promoting processes.

Experimentally, we were able to confirm the clinical relevance of elevated glycolysis and increased lactic acid production in various advanced PCa patient-derived xenograft (PDX) models and patient tumours using a novel metabolic pathway score. In particular, NEPC tumours appear to rely much more heavily on elevated aerobic glycolysis and MCT4-mediated lactic acid secretion. In a proof-of-concept study using MCT4-specific antisense oligonucleotides (ASOs), reduced MCT4 expression is able to reduce proliferation, invasion/migration, and glucose metabolism of advanced PCa cells *in vitro*. More importantly, we demonstrated in two distinct *in vivo* models containing residual functional immune cells that MCT4 inhibition enhanced anticancer immunity. Finally, a state-of-the-art *in silico* drug discovery pipeline was employed in the first steps towards developing a potent and specific MCT4 small molecule inhibitor. Computer modeling of MCT4 structure, virtual molecular docking, and downstream experimental validation identified a promising hit series based on the chemical scaffold of VPC-25009 as a potential second therapeutic modality for MCT4 inhibition.

Taken together, we were able to provide experimental support for our novel hypothesis regarding the central tumour-promoting and immunosuppressive role of cancer-generated lactic acid. A therapeutic approach blocking lactic acid secretion by targeting MCT4 function could thus inhibit multiple downstream lactate-associated processes for effective treatment of advanced PCa and other highly glycolytic cancers.

Lay Summary

Late-stage, therapy-resistant prostate cancer (PCa) remains a difficult-to-treat disease that urgently needs better therapeutics. We believe a new understanding of why cancer cells use energy differently than normal cells can help develop better treatment options. Although a patient's immune system is normally able to eliminate cancer cells, too much lactic acid produced by the tumour can prevent the immune system from working properly. We showed that this increased lactic acid production is common to advanced PCa tumours. Furthermore, inhibition of lactic acid transport from the key channel protein MCT4 could stop cancer cell growth and re-stimulate an anticancer immune response. We also used a state-of-the-art computer programming approach to design new drugs that inhibit MCT4 function. We believe that targeting cancer's unique characteristic of transporting lactic acid out of cancer cells into the surrounding tumour environment can become an effective therapeutic strategy for treating advanced PCa and other late-stage cancers.

Preface

Patient tumour samples were obtained following informed consent according to protocols approved by the Clinical Research Ethics Board of the University of British Columbia (UBC), the Vancouver Coastal Health Research Institute (VCHRI), and the BC Cancer Agency (BCCA). Animal care and experimental procedures were carried out following the guidelines of the Canadian Council on Animal Care (CCAC) as approved by the Animal Care Committee of UBC. The certificate numbers are H09-01628 and H04-60131 for the UBC Ethics Board, V09-0320 and V07-0058 for VCHRI, and A11-0275 and A15-0152 for animal protocols.

Sections of Chapter 1 have been published in Choi SY, Collins CC, Gout PW, Wang Y, Cancer-generated lactic acid: a regulatory, immunosuppressive metabolite? J Pathol, 2013. 230(4): 350-5 and Choi SY, Lin D, Gout PW, Collins CC, Xu Y, Wang Y, Lessons from patient-derived xenografts for better in vitro modeling of human cancer. Adv Drug Deliv Rev, 2014. 79-80: 222-37. I was the main author involved in the writing of the manuscripts. Gout PW provided critical reviews of the manuscripts. Wang Y supervised the writing process.

A section of Chapter 2 has been published in Lin D, Ettinger SL, Qu S, Xue H, Nabavi N, Choi SY et al., Metabolic heterogeneity signature of primary treatment-naive prostate cancer. Oncotarget, 2017. 8(16): 25928-41. I was involved in compiling the gene list used in calculating the metabolic pathway scores and assessing the glycolytic signature. Wang Y supervised the study and the writing process. Another portion of Chapter 2 and a section of Chapter 3 are currently being prepared as a manuscript for publication as Choi SY, Ettinger SL, Lin D, Xue H, Ci X, Nabavi N et al., Targeting MCT4 to reduce lactic acid secretion and glycolysis for treatment of neuroendocrine prostate cancer. I was the lead investigator responsible for experimental design, data analysis, and manuscript composition. Ettinger SL and Lin D conducted the bioinformatics analysis. Gout PW provided critical reviews of the manuscripts. Wang Y supervised the study and the writing process.

A version of Chapter 3 has been published in Choi SY, Xue H, Wu R, Fazli L, Lin D et al., The MCT4 gene: a novel, potential target for therapy of advanced prostate cancer. Clin Cancer Res., 2016.

22(11): 2721-33. I was the lead investigator responsible for experimental design, data analysis, and manuscript composition. Fazli L and Lin D scored and analyzed the immunohistochemistry staining of patient tumour samples. Gout PW provided critical reviews of the manuscripts. Wang Y supervised the study and the writing process. Portions of Chapter 3 were also included in an international patent application. Title: Monocarboxylate transporter 4 (MCT4) antisense oligonucleotide (ASO) inhibitors for use as therapeutics in the treatment of cancer (PCT/CA2016/000296). I am one of the inventors and conducted the experiments, analyzed the data, and interpreted the results. Wang Y is another inventor involved in the overall supervision of the study.

Portions of Chapter 4 were conducted in collaboration with Cherkasov A and Hsing M. They developed the *in silico* simulations and employed them for MCT4 structural modeling and small molecule inhibitor discovery. I was the lead investigator for the experimental assessments. I designed the screening techniques, collected and analyzed the experimental data, and interpreted the results. Wang Y provided overall supervision for the study.

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List of Abbreviations

&	and
Å	angstrom
°C	degrees Celsius
>	greater than
<	less than
µg	microgram
µm	micrometre
µM	micromolar
#	number
%	percent
±	plus or minus
×	times
17βHSD	17 beta-hydroxysteroid dehydrogenase
2MOE	2'-O-methoxyethyl
3βHSD	3 beta-hydroxysteroid dehydrogenase
3D	three dimensional
7ACC	7-aminocarboxycoumarin
A	adenosine
ABL	Abelson murine leukemia viral oncogene
aCGH	array comparative genomic hybridization
ACT	α 1-antichymotrypsin
ADMET	absorption, distribution, metabolism, excretion, toxicity
ADT	androgen deprivation therapy
AKT	AKT serine/threonine kinase
AL	Alabama
AM	acetomethoxy
ANOVA	analysis of variance
APC	adenomatous polyposis coli
AR	androgen receptor
ARE	androgen responsive element
ARG1	arginase 1
ASO	antisense oligonucleotides
ATCC	the American Type Culture Collection
ATP	adenosine triphosphate
AURKA	aurora kinase A
AZ	Arizona
BC	British Columbia
BCA	bicinchoninic acid
BCCA	British Columbia Cancer Agency

BCCRC	British Columbia Cancer Research Centre
BCL-2	B-cell leukemia/lymphoma 2 apoptosis regulator
BCR	breakpoint cluster region protein
BLAST	basic local alignment search tool
BPH	benign prostatic hyperplasia
BRAF	v-Raf murine sarcoma viral oncogene homolog B
C	cytidine
CA	California
CCAC	Canadian Council on Animal Care
CD147	cluster of differentiation 147
CD3	cluster of differentiation 3
CD31	cluster of differentiation 31
CD45	cluster of differentiation 45
CD8	cluster of differentiation 8
CD80	cluster of differentiation 80
CDK4	cyclin-dependent kinase 4
cDNA	complementary deoxyribonucleic acid
cEt	2’-4’ constrained ethyl
CGA	chromogranin A
CHC	α-cyano-4-hydroxycinnamate
CK14	cytokeratin 14
CK18	cytokeratin 18
CK19	cytokeratin 19
CK5	cytokeratin 5
CK8	cytokeratin 8
Cl	chloride
C-MYC	MYC proto-oncogene, bHLH transcription factor
CO_2	carbon dioxide
CoA	coenzyme A
cPSA	complexed prostate-specific antigen
cRNA	complementary ribonucleic acid
CRPC	castration-resistant prostate cancer
CT	computed tomography
CTLA-4	cytotoxic T-lymphocyte associated protein 4
CYP17A	cytochrome P450 family 17 subfamily A
DAB	3,3'-diaminobenzidine
DEK	DEK proto-oncogene
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone-sulphate
DHT	dihydrotestosterone
DIDS	4,4’-diisothiocyanatostilbene-2,2’-disulphonate

DLX1	distal-less homeobox 1
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dr.	doctor
DRE	digital rectal examination
EAAC1	excitatory amino-acid carrier 1
ECACC	the European Collection of Authenticated Cell Cultures
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EM	electron microscopy
EMT	epithelial-to-mesenchymal transition
ENO1	enolase 1
ENO2	enolase 2
ERG	ETS transcription factor
ERK	extracellular signal–regulated kinases
ETS	E26 transformation-specific
ETV1	ETS variant 1
FASN	fatty acid synthase
FBS	fetal bovine serum
FDA	Food and Drug Administration
FDG	^{18}F-fluorodeoxyglucose
FEP	free energy perturbation
FGF	fibroblast growth factor
fPSA	free prostate-specific antigen
G	guanosine
G_0	resting phase
G_1	gap 1 phase
G6PDH	glucose-6-phosphate dehydrogenase
GEM	genetically engineered mice
GLUT1	glucose transporter 1
GPS	genomic prostate score
GPU	graphics processing unit
GSTP1	glutathione S-transferase pi 1
GSTpi	glutathione S-transferase pi
H&E	hematoxylin and eosin
HBSS	Hanks' balanced salt solution
HOXC6	homeobox C6
hr	hour
HSP27	heat shock protein 27
IC_{50}	half maximal inhibitory concentration
ICAM-1	intercellular adhesion molecule 1

IDH1	isocitrate dehydrogenase 1
IDH2	isocitrate dehydrogenase 2
IDO	indoleamine-pyrrole 2,3-dioxygenase
IFNγ	interferon gamma
IGF-1	insulin like growth factor 1
IHC	immunohistochemistry
IL	Illinois
IL-6	interleukin 6
IL-8	interleukin 8
IL-10	interleukin 10
KEGG	Kyoto Encyclopedia of Genes and Genomes
Ki-67	marker of proliferation Ki-67
KRAS	Kirsten rat sarcoma viral oncogene homolog
L701H	leucine-701 to histidine
LADY	LPB-Tag transgenic mice
LDHA	lactate dehydrogenase A
LDHB	lactate dehydrogenase B
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
log2	binary logarithm
LTL	the Living Tumour Laboratory
M2	type II macrophage
MA	Massachusetts
mAAT	mitochondrial aspartate aminotransferase
MCT	monocarboxylate transporter
MCT1	monocarboxylate transporter 1
MCT10	monocarboxylate transporter 10
MCT2	monocarboxylate transporter 2
MCT3	monocarboxylate transporter 3
MCT4	monocarboxylate transporter 4
MCT8	monocarboxylate transporter 8
MD	Maryland
MD	molecular dynamics
MDSC	myeloid-derived suppressor cells
mg	milligram
MHC	major histocompatibility complex
MiPS	Mi-prostate Score
mL	millilitre
mM	millimolar
mm	millimetre
mm^3	cubic millimetre

MMP	matrix metalloproteinase
mo	month
MOE	molecular operating environment
mPIN	mouse prostatic intraepithelial neoplasia
MQH_2O	Milli-Q water
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSKCC	Memorial Sloan Kettering Cancer Center
mTOR	mechanistic target of rapamycin kinase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MYCN	MYCN proto-oncogene, bHLH transcription factor
n	sample size
Na	sodium
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NDRG3	N-myc downstream regulated gene family member 3
NE	Nebraska
NE	neuroendocrine
NEPC	neuroendocrine prostate cancer
NFκB	nuclear factor kappa B
NFAT	nuclear factor of activated T cells
ng	nanogram
NHE1	Na^+/H^+ hydrogen exchanger 1
NHT	neoadjuvant hormone therapy
NIV	New International Version
NJ	New Jersey
NK	natural killer
NK1.1	killer cell lectin-like receptor subfamily B, member 1
NKX3.1	NK3 homeobox 1
nM	nanomolar
nm	nanometre
NOD/SCID	nonobese diabetic/severe combined immunodeficiency
nt	nucleotide
NY	New York
ON	Ontario
p	p-value
p63	tumor protein p63
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PCa	prostate cancer

PCA3	prostate cancer antigen 3
pCMBS	p-chloromercuribenzenesulfonic acid
PDB	the Protein Data Bank
PDK1	pyruvate dehydrogenase kinase 1
PD-L1	programmed cell death 1 ligand 1
PDX	patient-derived xenograft
PEG10	paternally expressed 10
PET	positron emission tomography
PGAM1	phosphoglycerate mutase 1
PGK1	phosphoglycerate kinase 1
pH	potential of hydrogen
PHI	prostate health index
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PS	phosphorothioate
PSA	prostate-specific antigen
pT	pathologic tumour
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidene fluoride
QC	Quebec
qPCR	quantitative polymerase chain reaction
QSAR	quantitative structure–activity relationship
RAF	RAF-1 proto-oncogene, serine/threonine kinase
RANKL	receptor activator of nuclear factor kappa-B ligand
RASSF1	Ras association domain family member 1
RB	retinoblastoma transcriptional corepressor 1
RIPA	radioimmunoprecipitation assay
RMSD	root-mean-square deviation
RNA	ribonucleic acid
RNase	ribonuclease
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
siRNA	small interfering ribonucleic acid
SLC16A1	solute carrier family 16 member 1
SLC16A10	solute carrier family 16 member 10
SLC16A2	solute carrier family 16 member 2
SLC16A3	solute carrier family 16 member 3
SLC16A7	solute carrier family 16 member 7
SLC16A8	solute carrier family 16 member 8
SMI	small molecule inhibitor
SMN2	survival of motor neuron 2, centromeric

SNAIL	zinc finger protein, snail family transcriptional repressor
SPINK1	serine peptidase inhibitor, Kazal type 1
SPOP	speckle type BTB/POZ protein
SRD5A	steroid 5 alpha-reductase
SRC	Rous sarcoma oncogene, non-receptor tyrosine kinase
SRE	skeletal-related events
SREBP	sterol regulatory element-binding protein
STAT3	signal transducer and activator of transcription 3
SV40	Simian vacuolating virus 40
SYP	synaptophysin
T	thymidine
T877A	threonine-877 to alanine
TCA	tricarboxylic acid
TCR	T cell receptor
TGFβ	transforming growth factor beta
TMA	tissue microarray
TMPRSS2	transmembrane protease, serine 2
TNM	tumour, node, metastasis
TP53	tumor protein p53
TRAMP	transgenic adenocarcinoma of the mouse prostate
TRUS	transrectal ultrasound
TX	Texas
UBC	the University of British Columbia
ud	undetected
US	the United States
USA	the United States of America
UT	Utah
VA	Virginia
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VHL	von Hippel-Lindau tumor suppressor
VPC	Vancouver Prostate Centre
vs	versus
WB	western blot
WI	Wisconsin
YZ	Dr. Yuzhuo Wang
ZIP1	zinc transporter ZIP1

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To the Lord Most High

My God, from now until time is no more

Here I raise an altar to Your steadfast love and faithfulness

May this be but another one of many testaments to You my Shepherd

Thus concludes my journey through Deuteronomy 8:2-6

Chapter 1: Introduction

1.1 Prostate Cancer

1.1.1 The Prostate

The prostate gland is a walnut-sized male accessory sex organ surrounding the urethra directly below the bladder. It is divided into three distinct glandular regions: the peripheral zone, which is closest to the rectum and accounts for approximately 70% of the glandular volume; the central zone, which surrounds the ejaculatory ducts and makes up around 25% of the prostate; and the transition zone, which surrounds the urethra [1, 2]. The prostate's primary physiological function is the secretion of prostatic fluid for the protection and nourishment of spermatozoa in the ejaculate. This is done from pseudostratified columnar epithelium forming a two-cell layer of outer low cuboidal basal cells beneath tall columnar luminal secretory cells. This epithelium is also interspersed with rare neuroendocrine cells and is embedded in fibromuscular stroma, ultimately forming tubuloalveolar glands radiating from the urethra [3, 4].

Developmentally, the prostate originates from the urogenital sinus and fully matures after puberty. The initial embryonic stages of prostate development are highly dependent upon signaling from circulating androgens produced by the fetal testes [5]. Early studies using tissue recombination strategies have demonstrated that the androgens act predominantly through the urogenital mesenchyme to induce epithelial differentiation [6]. Urogenital epithelium in the presence of androgen-insensitive mesenchyme resulted in the development of vaginal tissue, while urogenital epithelium regardless of androgen sensitivity developed into prostatic tissue in the presence of wild-type mesenchyme. It is only in the final stages of morphogenesis and initiation of secretory functions that androgen signaling is necessary in the epithelium [7]. Cellularly, early prostatic epithelial cells co-express lineage markers of both luminal and basal cells (CK8/CK18 and CK14/CK5/p63/CK19/GSTpi respectively) prior to differentiation. A small proportion of these cells remain in the adult prostate as stem/progenitor cells, residing in the basal layer of the mature prostate epithelium [8].

1.1.2 Prostate Cancer and Progression

Prostate cancer (PCa) is the most commonly diagnosed non-cutaneous cancer in North American males and is a leading cause of cancer deaths [9]. It is the second-most commonly diagnosed cancer in males worldwide, with 1.1 million new cases recorded in 2012 [10]. The latest statistics from the Canadian Cancer Society in 2016 estimated that 21,600 men would be diagnosed with PCa, representing 21% of all new cancer cases in men in Canada. Furthermore, they estimated that 4,000 men would die from PCa, representing 10% of all cancer deaths in Canadian men [11]. With approximately 1 in 8 men developing PCa within their lifetime, it is unsurprising that clinical management of the disease is a significant health care and economic burden [12]. As such, improved testing and better treatment options are active areas of research.

The vast majority of PCa cases arise from the peripheral zone of the prostate and present overwhelmingly as acinar adenocarcinoma [1, 2], with a number of documented rare histological variants including mucinous adenocarcinoma, ductal adenocarcinoma, and neuroendocrine carcinoma [13]. All PCa adenocarcinomas can be considered androgen-dependent and reliant on the androgen receptor (AR) signaling axis for growth and survival. However, the near-universal common histology and reliance upon the AR pathway does not reflect the well-known heterogeneity of PCa. Clinically, PCa is often a multi-focal disease, with cancerous tissue originating from multiple locations [14] and exhibiting distinct pathological characteristics such as metastatic potential [15, 16]. Furthermore, for reasons that are still poorly understood, a subset of PCa is considered high-risk, progressing rapidly and is highly aggressive and therapy-resistant. Other cases, however, remain indolent for extended periods of time [17]. This heterogeneity also extends to the molecular level. Over 50% of PCa harbour the recurrent TMPRSS2-ETS gene rearrangement, where the 5’ regulatory elements of the androgen-responsive TMPRSS2 is fused with a member of the ETS family of oncogenic transcription factors (such as ERG and ETV1), partially driving disease progression [18, 19]. Additionally, SPINK1 overexpression and SPOP mutations are also commonly observed in 10-15% of PCa and seem to be mutually exclusive of ETS-gene rearrangements, representing a separate subtype of PCa [20, 21]. Finally, NKX3.1 deletions, PTEN inactivation, TP53

mutation, and RB loss are also common genetic aberrations observed in PCa and can collectively involve between 40-60% of PCa tumours [22-24].

The majority (~92%) of patients with PCa are first diagnosed with local or regional disease. Localized disease is completely confined within the prostate, while regional disease have cancer that has spread into nearby areas such as invasion into the prostatic capsule or dissemination into the seminal vesicles and pelvic lymph nodes. Collectively, patients with localized or regional disease have a 5-year survival rate of almost 100% [9]. However, a minority of patients can present with or progress towards PCa with distant metastasis, of which the bone is by far the most common metastatic site. Visceral metastasis into organs such as lungs, liver, adrenals, and brain can also occur, albeit at a much lower frequency [25]. The 5-year survival for PCa patients initially diagnosed with metastatic disease is only 29% [9]. As such, the clinical management of these metastatic PCa remains a major challenge, particularly with respect to the development of treatment resistance. The development of novel, more effective therapeutics is still very much needed.

1.1.3 Diagnosis and Clinical Stages

Although population-wide screening for PCa is not recommended due to difficulties in balancing potential benefits with the risk of overdiagnosis and harmful side-effects of subsequent treatments [26], a number of factors may lead patients and physicians to suspect the presence of PCa and perform diagnostic tests. Common symptoms of early-stage PCa include hematuria and difficulty with urination due to prostate enlargement, while symptoms of late-stage PCa that has potentially metastasized include bone pain in the lower back region, weight loss, anemia, and incontinence [27]. The detection of abnormal prostate growth by digital rectal examination (DRE) during routine physical examinations together with elevated levels of prostate-specific antigen (PSA) in the serum have also led to the discovery of PCa in many asymptomatic patients [28]. However, a number of other conditions, such as benign prostatic hyperplasia (BPH) and prostatitis, can also contribute to abnormal DRE and elevated serum PSA. As such, histopathological evaluation through biopsy is still required for definitive diagnosis [28].

Typically, abnormal DRE, serum PSA levels >4 ng/mL, or rising PSA levels >0.75 ng/mL/year calculated from at least three PSA determinations over 18 months are considered pathological and at higher risk of PCa [29]. Transrectal ultrasound (TRUS)-guided needle biopsy is usually recommended for patients falling under these categories, and 10 to 12 cores are taken from the whole prostate for a representative sample, with additional cores taken from any suspect regions [28, 29]. The Gleason classification system is the most commonly used for histopathological assessment of prostate biopsy samples. A number from 1 to 5 is used to describe the appearance of the prostate glands, with grade 1 being the most well-differentiated, consisting of small uniform glands most similar to normal prostate tissue, and grade 5 being the least differentiated, with only occasional glandular structures. Most prostate tumours are found beginning at grade 3, with cancer cells remaining relatively well-differentiated but have invaded noticeably into the surrounding prostate tissue. The grades corresponding to the two most common growth patterns in the biopsy sample are then added together to arrive at a final Gleason score and is associated with disease aggressiveness and prognosis. A Gleason score ≤ 6 is largely considered low risk while a Gleason score of 7 is considered of intermediate risk. The prognosis for patients with Gleason score 8 tumours are significantly worse, and becomes even more unfavourable for patients with Gleason score 9 and 10 tumours [30-32].

In addition to Gleason scores, PCa is also described by the tumour, nodes, metastasis (TNM) staging system. Although each tumour category can be further subdivided, they are broadly as follows: T1 tumours are not clinically apparent (i.e., not palpable by DRE and not visible through imaging) and are often found incidentally from other procedures or from biopsy due to elevated PSA levels; T2 tumours are confined to the prostate; T3 tumours have extended through the prostatic capsule and may have invaded the seminal vesicles; and T4 tumours have invaded into adjacent structures and organs beyond the seminal vesicles [33]. Regional lymph node involvement (N stage) for PCa is simply categorized by the presence or absence of cancer cells in the pelvic lymph nodes and is assessed by histology following lymphadenectomy [33, 34]. Finally, the M stage describes the presence or absence of metastasis in non-regional lymph nodes, bones, and other distant organs such as liver and lungs. As skeletal metastasis is

the most frequently observed metastatic event in PCa, bone scintigraphy using technetium-99m as a radiotracer is common practice. In select cases, pelvic computed tomography (CT) scan, magnetic resonance imaging (MRI) or ^{18}F-fluorodeoxyglucose (FDG)-positron emission tomography (PET) may also be used to assess metastatic nodules [28, 33, 34].

The combination of serum PSA, biopsy Gleason grade, and clinical stage have been used extensively in the diagnosis and risk stratification of PCa patients. However, significant limitations remain for this traditional approach, particularly in light of the common over-diagnosis and over-treatment of otherwise indolent disease [35, 36]. Recent advances in alternative diagnostic approaches have yielded some level of success, but none have yet entered widespread clinical practice. For example, certain refinements to the PSA test have been approved by the United States (US) Food and Drugs Administration (FDA) for diagnostic purposes in borderline cases to reduce unnecessary biopsies. The fraction of PSA bound to the serum protein ACT, known as complexed PSA (cPSA), can be distinguished from unbound free PSA (fPSA). This percent fPSA decreases in patients with PCa, making such measurements particularly useful for risk stratification in men with total PSA between 4 to 10ng/mL [37, 38]. Similarly, the prostate health index (PHI) measures total PSA, fPSA, and the [-2] proPSA isoform to calculate the likelihood of finding PCa, reducing the need to biopsy low-risk disease [39, 40]. The 4Kscore Test, measuring total PSA, fPSA, intact PSA, and human kallikrein 2, can also be used in conjunction with other clinical information to better predict the risk of finding aggressive PCa with Gleason score $\geq$7 [41-43].

In addition to PSA tests, other diagnostic markers have been employed or are under investigation. The FDA has approved the use of the PCA3 assay for determining whether repeat biopsies are necessary for patients with previous negative biopsies [44, 45]. In particular, a ratio comparing the levels of the long non-coding RNA PCA3 to PSA mRNA as measured in a post-DRE urine sample would indicate the likelihood of a positive biopsy result [46, 47], potentially sparing patients from biopsy-associated discomforts and complications. Alternatively, the Mi-Prostate Score (MiPS) measuring the TMPRSS2:ERG gene fusion product as a urinary biomarker in conjunction with PCA3 [48, 49] and the

SelectMDx urinary test measuring HOXC6 and DLX1 mRNA levels have both been demonstrated to predict the likelihood of high-grade PCa [50, 51]. On a tissue biopsy level, the ConfirmMDx epigenetic test measuring GSTP1, APC and RASSF1 by quantitative methylation specific polymerase chain reaction [52, 53] and the OncotypeDx Genomic Prostate Score (GPS) measuring RNA expression of a 17-gene panel [54, 55] can also similarly predict PCa aggressiveness. Taken together, these additional diagnostic and predictive indicators can supplement traditional clinical information from PSA and Gleason grade to better assist physicians and patients, providing greater guidance towards risk assessment and associated treatment decision-making.

1.1.4 Treatments

Because of the long natural history of early, localized PCa, over 90% of patients harbouring low-grade PCa remain alive even at 15 years post-diagnosis, with intervening deaths occurring primarily from PCa-unrelated causes [56-58]. As such, aggressive treatment of indolent, low-risk PCa subject patients to considerable side-effects without necessarily improving survival outcomes. An active surveillance approach is thus often adopted for these cases to avoid overtreatment, particularly for patients whose life expectancy is under 15 years [58]. Patients placed on active surveillance are monitored mainly by DRE and serial assessments of serum PSA levels. In the event of rising PSA, repeat biopsies and MRI can be done to determine whether the disease has progressed [28, 59-61]. Active treatment is initiated at signs of progression but can also be triggered by patient request [28, 62].

PCa patients who have progressed on active surveillance and those with higher-grade, localized disease can receive treatment with curative intent. The primary treatment modalities at this stage include radical prostatectomy, radiation therapy, and brachytherapy. Radical prostatectomy involves the surgical removal of prostate tissue and can be done either through open retropubic or prenial surgery or by laparoscopic surgery with or without robotic assistance. Similar post-operative outcomes are observed irrespective of surgical methods [63]. Despite the development of nerve-sparing surgical procedures, urinary incontinence and erectile dysfunction remain common side effects [28]. Radiation can also be

used to locally eradicate PCa cells. External-beam radiation therapy delivers radiation beams from multiple computer-calculated angles and deposits focused radiation doses at the prostate, while brachytherapy involves the direct implantation of small radioactive pellets into the prostate for the same therapeutic effect. However, due to the close proximity of other organs such as the bladder and intestines, common side effects of radiation therapy include gastrointestinal toxicity, genitourinary toxicity, urinary incontinence, and erectile dysfunction [64]. A small increased risk of developing secondary cancers in the region, such as bladder cancer and colorectal cancer, is also possible [65]. Because comparative studies between the efficacy of surgery and radiation have often suffered from poor trial design, and multi-study comparisons are further confounded by inconsistent risk-group stratifications between studies, these treatment modalities are currently considered largely comparable [66-68].

Despite treatment with curative intent, approximately 20-30% of patients treated for localized PCa experience biochemical recurrence (defined as a rise in PSA levels following treatment) with or without metastatic spread [69, 70]. In such cases, together with patients initially diagnosed with advanced disease, androgen deprivation therapy (ADT) is the first-line systemic treatment. PCa has been widely recognized as an androgen-sensitive disease since the 1940s [71]. In particular, PCa cell growth and proliferation, at least initially, is promoted through the canonical androgen signaling pathway. Leydig cells in the testes produce 90-95% of the circulating testosterone while the remaining 5-10% is produced by the adrenal glands [72]. The circulating testosterone is then converted in prostate tissues by 5-alpha-reductase (SRD5A) into dihydrotestosterone (DHT), which is 10-fold more active [73]. The androgens diffuse through the cell membrane and bind to AR, a cytoplasmic steroid hormone receptor and ligand-activated transcription factor. Upon ligand binding, AR homodimerizes, becomes phosphorylated, and translocates into the nucleus to bind to androgen-responsive elements (AREs) and initiate transcription of downstream genes involved in cell cycle regulation and proliferation [74, 75]. As such, the removal of androgens results in the involution of the prostate due to apoptosis of secretory epithelial cells and degeneration of prostatic blood vessels [76, 77]. Clinically, initial androgen deprivation results in near-universal PCa regression. This can be achieved either surgically by the removal of the testis

(orchiectomy) or pharmaceutically through administration of luteinizing hormone-releasing hormone (LHRH) agonists or antiandrogens. LHRH agonists such as leuprolide and goserelin stimulate LHRH receptors in the pituitary, causing the release of luteinizing hormone (LH), which in turn stimulates the gonadal production of sex steroid hormones. As such, treatment with LHRH agonists causes an initial LH surge and increased testosterone production from the testes. However, sustained stimulation of the LHRH receptor results in a feedback inhibition where LHRH receptor expression is downregulated, desensitizing the pituitary. This results in a decrease in LH production and release, ultimately ablating testicular testosterone production and reducing serum testosterone to castrate levels [78-80]. The suppression of androgen signaling can also be achieved with antiandrogens such as flutamide and bicalutimide. Antiandrogens bind to the ligand-binding pocket of AR and are competitive inhibitors, thus blocking androgen-steroid receptor interactions and ablating AR signaling [81, 82]. However, as androgen signaling is critically involved in a number of normal physiological processes, substantial side effects are observed for ADT and include decreased libido, erectile dysfunction, gynecomastia, osteoporosis, and anemia [70].

1.1.5 Treatment Resistance

Despite the initial therapeutic response to ADT, advanced PCa inevitably recurs in a treatment resistant form known as castration-resistant prostate cancer (CRPC). A number of mechanisms have been proposed as to how relapse from ADT occurs and, broadly speaking, can be divided into ligand-dependent and ligand-independent mechanisms [83]. Ligand-dependent mechanisms of castration resistance reactivate AR signaling through the binding of steroid hormone to AR even in the presence of castrate levels of serum testosterone. This can occur through the synthesis of DHT locally in the prostate using the adrenal precursor steroids dehydroepiandrosterone (DHEA) and its sulphate (DHEA-S) via 3βHSD, SRD5A, and 17βHSD [84], thus maintaining a sufficiently high level of intratumoural androgens to sustain AR signaling for PCa growth and survival [85, 86]. Alternatively, amplification and resultant overexpression of AR can lead to hypersensitivity towards androgens in PCa cells, allowing even low

levels of testosterone and DHT to activate transcription of downstream genes [87-89]. Finally, mutations in the AR ligand-binding domain also allow the promiscuous activation of AR in the presence of alternative steroids such as glucocorticoids, progesterone, estradiol and antiandrogens [90-93]. For example, the T877A mutation found in the LnCaP cell line enlarges the ligand binding pocket and accommodates side chains found on progesterone and cortisol [91, 94], while the L701H AR mutant form favourable hydrogen bonds with the 17-alpha-hydroxyl group in these other steroid hormones [95].

Ligand-independent mechanisms can also facilitate PCa escape from ADT. One way by which AR signaling is sustained in the absence of androgens is through alternative AR splice variants. In particular, ARv1, ARv7, and ARv567 are truncated and remain constitutively active due to the missing C-terminal ligand-binding domain [96-98]. Alternatively, the AR-signaling pathway can be activated by signaling through other growth-promoting pathways. For example, downstream signaling cascades from growth factors such as IGF-1/FGF/EGF [99-101], cytokines such as IL-6/TGFβ [102-104], and the PTEN/PI3K/AKT signaling axis [105-108] can converge on various aspects of AR phosphorylation, dimerization, and localization to the nucleus to sustain AR activation. Other survival and proliferative mechanisms bypassing the AR-signaling pathway have also been associated with development of castration resistance. The overexpression of the anti-apoptotic protein BCL-2 has been shown to protect PCa cells from cell death following androgen deprivation [109-111], while amplification of the C-MYC transcription factor and resultant overexpression have also been commonly observed in CRPC patients [112, 113], suggesting that it can promote androgen-independent PCa growth by its role as a proto-oncogene and master regulator of cell proliferation [114-116].

Finally, cellular mechanisms of castration resistance have also been proposed. ADT have been shown to induce an epithelial-to-mesenchymal transition (EMT) in PCa cells, potentially enhancing their migratory potentials and facilitating progression to metastasis [117-119]. Furthermore, the presence of androgen-independent PCa cells even before the initiation of ADT could theoretically allow PCa cells to repopulate in the absence of androgens. Although clonal expansion of these cells is possible, recent studies have largely redefined these “lurker” cells as PCa stem cells by use of a plethora of stem cell

markers, focusing on their stem-like characteristics such as the capacity for self-renewal and tumour initiation in progression towards castration resistance [120-123]. More significantly, however, is the potential for androgen-dependent PCa adenocarcinoma cells to transdifferentiate into androgen-independent neuroendocrine PCa (NEPC) cells [124, 125]. NEPC is characterized as histologically resembling small cell carcinoma, lacking conventional androgen-dependent PCa markers such as AR and PSA, and expressing typical neuroendocrine (NE) markers such as synaptophysin (SYP) and chromogranin A (CGA) [126, 127]. Although *de novo* incidences of NEPC are extremely rare and account for only 0.3-1% of all diagnosed PCa cases [128], it is estimated that its prevalence may be much higher in the later stages of disease progression, potentially comprising up to 25% of CRPC as second-generation antiandrogens enter widespread clinical use [125].

1.1.6 Other Systemic Treatments for Advanced PCa

From a clinical management perspective, if CRPC remains androgen-sensitive, recently-approved second-generation inhibitors of the AR signaling pathway could be used to induce a second round of remission. These inhibitors include the AR inhibitor enzalutamide [129, 130] and the CYP17A inhibitor abiraterone acetate, which inhibits androgen biosynthesis [131, 132]. However, despite initial efficacy, the renewed targeting of the AR signaling axis is still not a curative therapeutic strategy, and a number of resistance mechanisms have already been proposed and observed in the clinic, including point mutations of the AR ligand binding domain [133], the expression of constitutively active AR variants such as ARv7 [134, 135], and transdifferentiation into androgen-independent NEPC [127, 136].

In cases where CRPC is unresponsive to or has progressed from secondary hormone therapy, only a limited number of marginally effective therapeutic options remain. Historically, the cytotoxic agents estramustine, which disrupts microtubule dynamics via direct binding to tubulin [137, 138], and mitoxantrone, a DNA intercalating agent inhibiting DNA synthesis by inducing covalent topoisomerase II-DNA complexes [139], have been used with modest effects. The current standard of care, docetaxel plus prednisone, only improves overall survival from historical treatments by a few months, albeit with

significant pain and quality of life benefits [140]. Unfortunately, progression from chemotherapy is observed in all patients within 6-8 months [141]. While docetaxel can effectively inhibit mitosis by promoting tubulin assembly and inhibiting microtubule depolymerisation [142, 143], multiple resistance mechanisms have been described [144], including the expression of multidrug resistance proteins such as p-glycoprotein to facilitate drug efflux [145, 146] and the utilization of alternate tubulin isoforms [147]. More recently, the second-generation taxane cabazitaxel, which has lower affinity to p-glycoprotein [148], has been approved for CRPC patients who have progressed from docetaxel therapy [149].

Besides systemic chemotherapy, other currently approved therapeutics for clinical management of advanced PCa include the cancer vaccine sipuleucel-T and bone metastasis-targeting agents such as zoledronic acid, denosumab, and radium-223. Sipuleucel-T is manufactured from the patient's own immune cells and is the first cancer vaccine approved by the US FDA [150, 151]. As an active cellular immunotherapy, the patient's antigen presenting cells are first activated and T cells primed *ex vivo* to recognize and eliminate PCa cells expressing prostatic acid phosphatase when reintroduced into the body [152]. PCa metastasis to the bone often disrupts the normal equilibrium between bone resorption from osteoclasts and bone formation from osteoblasts, resulting in an overall loss of bone integrity. This causes a number of related complications including pathological fractures, spinal compressions, and bone-directed surgery or radiation therapy, collectively classified as skeletal-related events (SRE) [153]. Zoledronic acid is a bisphosphonate that is deposited in the bone matrix following administration, inhibiting osteoclast activity and reducing SREs in CRPC patients [154, 155]. Alternatively, the monoclonal antibody denosumab targets RANKL and its downstream activation of osteoclasts, restoring bone mineral density and delaying onset of bone metastasis in CPRC patients [156, 157]. Finally, radium-223 is an alpha-particle emitter that preferentially localizes to sites of high bone turnover due to its similarity to calcium [158]. It can thereby deposit therapeutic doses of radiation at the site of bone metastasis, alleviating bone pain and extending survival in CPRC patients [159-161]. Similar to chemotherapy, none of these treatment options are considered curative. In particular, bone-targeting

agents are often used only with the palliative intent of alleviating pain and reducing bone metastasis-related complications [162].

It is worth noting, however, that the above description of clinical progression and associated management options represent a gross simplification for summary purposes. Clinical considerations are often more complicated and do not progress in linear fashion. Depending on patient situations, various "second-line" therapeutic modalities may be considered comparable first-line treatment options even in hormone-naive patients. In particular, the therapeutic benefits of first-line docetaxel and abiraterone have already been demonstrated in clinical trials [163-166]. Additionally, the appropriate selection of front-line therapy for CRPC patients and the proper sequence of drug administration remain an active area of debate. The limited data availability for various post-treatment settings and a lack of head-to-head clinical trials have made evidence-based treatment decisions difficult, further adding to the complexity of the current standard of care [167-169].

With regard to NEPC, it is a highly aggressive disease with a median survival of less than one year [125, 170]. There is currently no consensus standard of care, with platinum-based chemotherapies such as cisplatin and carboplatin being suggested based on NEPC's possible similarities to other small cell cancers [171, 172]. However, more stringent clinical trials have yet to demonstrate any notable improvement of survival for this approach [173-176]. A number of other potential therapeutic targets have been suggested, including AURKA, MYCN, DEK, and PEG10 [127, 177, 178], but remain in various preclinical stages. As such, the rarity of NEPC prior the advent of second-line hormone therapies and our limited understanding of its biology make the identification of effective therapeutic options highly challenging at this point in time.

Taken together, advanced PCa including CRPC and NEPC remain a challenging disease to manage in the clinic. Although targeting the AR signaling axis with second-generation antiandrogens appears to be the most promising, particularly in cases where CRPC remains androgen-dependent, multiple mechanisms of treatment resistance make sustained remissions unlikely. Therapeutic options beyond enzalutamide and abiraterone for advanced PCa remain limited and marginally effective. More

critically, increasing prevalence of the lethal NEPC subtype in the clinic following progression from secondary ADT further restricts potential therapeutic options. A number of clinical trials are ongoing to investigate the efficacy of experimental inhibitors against both known and novel therapeutic targets in CRPC [179, 180]. These include the AR inhibitors EPI-001 [181] and ARN-509 [182], the PI3K/AKT/mTOR pathway inhibitors AZD5363 [183] and ipatasertib [184], the PARP inhibitor olaparib [185], the AURKA inhibitor alisertib [186], and the immunotherapies PROSTVAC [187] and DCVAC [188]. While initial results may be promising for a number of these experimental therapies, an expanded panel of effective therapeutics remain much needed for the clinical management of advanced PCa. In particular, as the prevalence of truly androgen-independent subtypes of CRPC are expected to increase, the identification of therapeutic targets outside the AR signaling axis will become more clinically relevant.

1.2 Preclinical Models of Prostate Cancer

Prostatic carcinogenesis, PCa progression, and response to therapy involve a series of highly complex biological processes influenced by a plethora of pertinent factors. While direct manipulations on PCa patient populations would provide the most clinically relevant information to help better understand disease biology and identify effective therapeutic options, such experimentations are often ethically, logistically, and economically prohibitive. As such, much of our current understanding and ongoing investigations towards PCa, including the identification and validation of potential novel therapeutic targets, rely heavily on well-established experimental models. Cell-based systems have greatly improved our understanding of molecular mechanisms underlying various cellular processes pertinent to PCa survival and proliferation, and are fundamental in particular to identifying critical signaling pathways and investigating relevant pharmacological inhibitors [189]. Animal-based models have also enabled us to understand early events during prostatic carcinogenesis, PCa progression, and discover crucial functions of the tumour microenvironment, and are an indispensible component of preclinical evaluations of drug efficacy [190]. However, as models commonly used in PCa research generally simplify the complex

disease state into experimentally manageable representations, certain clinically relevant aspects are inevitably lost or down-played. As such, no single model can fully recapitulate PCa as observed in the clinic, and it is important to recognize the inherent limitations associated with balancing ease-of-use for experimental manipulations and fidelity in mimicking the original disease.

1.2.1 Cell Lines

PCa cell lines represent the easiest and most commonly used model to mimic patient PCa in an experimental setting. Traditionally, *in vitro* work investigating PCa primarily employed three cell lines, i.e. PC-3, DU-145, and LNCaP [189, 191]. PC-3 cells were derived from a vertebral metastasis [192] while DU-145 cells were derived from a brain metastasis [193]. Both cell lines exhibit androgen-independent growth and do not express AR. LNCaP cells were derived from lymph node metastasis [194] and remains androgen sensitive, albeit expressing a mutant AR. Beyond these three cell lines and their various derivatives, other commonly used PCa cell lines include those with xenograft origins such as 22Rv1. Meanwhile, there are a few benign cell lines immortalized by transgene insertions such as BPH-1 and RWPE-1 [195]. The NEPC cell line NCI-H660 has also been described [196, 197], and more recently, the enzalutamide-resistant cell line MR49F was also developed from an enzalutamide-resistant LNCaP tumour [198].

Despite its ease of use, the cell line model of PCa has a number of significant drawbacks. Of the limitations specific to PCa, the highly proliferative and aggressive nature of available PCa cell lines stand in direct opposition to clinical PCa, the vast majority of which remain indolent or slow-growing for extended periods of time. In a related manner, the indolent or slow-growing nature also makes cell line development exceptionally difficult. Consequently, compared to cancers originating from other organs, the spectrum of common clinical PCa phenotypes and genetic characteristics are not well represented in the restricted number of available cell lines [199]. Of drawbacks more broadly applicable to cell cultures, PCa cell lines are highly homogenous cell populations arrayed along a two-dimensional surface during experimentation. This poorly reflects both the three-dimensional structure of a patient tumour and key

interactions with other tumour-associated cell types [200]. Additionally, the decades-long serial propagation of PCa cell lines have led to problems associated with cross-contamination and misidentification [197], as well as accumulation of mutations and other genetic alterations, further diminishing their ability to faithfully represent patient PCa. One strategy to overcome these common deficiencies of *in vitro* PCa cell line models is the use of organoid cultures. By using various scaffolds such as inserts, supportive matrices, co-cultured stromal cells, and multicellular aggregates [201, 202], a three-dimensional structure that supports the growth of various cell types in an organized manner can recapitulate original tissue histology at a basic level [203]. In particular, cancerous prostatic structures mirroring original tumour histology can be recreated for applications in drug screening, cell lineage analysis, and understanding contributions of various tumour microenvironmental factors to disease progression [204, 205].

1.2.2 Genetically Engineered Mouse (GEM) Models

While recent developments in prostatic organoid cultures have significantly advanced our ability to better mimic PCa tumours *in vitro*, the complex interactions between cancer cells and the surrounding environment are still not fully accounted for. As the patient tumour microenvironment affects cancer growth and progression via contributions from multiple factors including, but not limited to, immune cells, fibroblasts, paracrine signals, nutrient supplies, and oxygen gradients, animal models can better recapitulate most of these factors in a more native environment and are a mainstay of current PCa research [200].

One approach to modeling PCa in animals is the use of genetically engineered mice (GEM). They are particularly useful for investigating the contributions of various genetic alterations to the process of carcinogenesis. In the majority of these models, expression of specific oncogenes or of the Cre recombinase to knockout tumour suppressors is specifically targeted to prostatic tissue by the use of prostate specific promoters such as the probasin promoter. Early efforts have used the oncogenic viral SV40 T antigen to promote PCa development in mice, and both the LADY [206] and TRAMP [207]

models of PCa have been widely used in numerous studies. However, despite the relative ease of generating these models and the high penetrance observed, the SV40 T antigen is an exogenous oncogene considered not relevant to the development of human PCa [208]. Alternatively, as our understanding of the molecular mechanisms involved in PCa development increased, more relevant genetic aberrations have been identified and used to generate GEM models of PCa. Some common pathway aberrations that have been demonstrated to lead to *de novo* PCa development in mice include activation of PI3K/AKT signaling in Pten knockout mice [209]. The process of carcinogenesis can be further accelerated by complementing Pten loss with additional alterations in mice such as Tp53 deletion [210], K-ras expression [211], Myc expression [212], and Erg expression [213].

While these GEM models can reliably give rise to PCa in mice, a number of caveats pertaining to differences between human and mouse prostates need to be considered for more accurate interpretation of experimental results. For example, mouse prostate is anatomically distinct from human prostate, with the former consisting of four separate lobes that do not necessarily mirror the four prostatic zones found in humans [208]. Furthermore, PCa is generally not a disease that spontaneously occurs in mice. As such, most GEM models develop PCa during sexual maturation rather than advanced age as in humans and could signify important underlying biological differences [190, 214]. Finally, a number of pathological conditions other than PCa may present in the mouse prostate following genetic manipulation. These cellular proliferations could range from benign non-neoplastics hyperplasia to mouse prostatic intraepithelial neoplasia (mPIN) to malignant carcinoma [215]. As such, careful histological evaluations together with better awareness of distinctions between human and mouse prostate are required to avoid misinterpreting results from GEM models of PCa.

1.2.3 Patient-derived Xenograft (PDX) Models

The major concern that GEM models of PCa are of mouse origin and thus may not accurately reflect human PCa can be alleviated by grafting human PCa cells into mice. Traditionally this has been done by subcutaneously injecting cultured human cancer cell lines into immunodeficient mice to allow

tumour formation for experimentation. However, serious limitations have rendered the widespread use of this methodology problematic. Recent analyses of translational cancer research have revealed that less than 8% of positive preclinical testing using animal models translate into successful clinical trials [216, 217]. This high failure rate can be attributed, at least in part, to the lack of clinical relevance in traditional xenograft models. In addition to issues associated with cell lines regarding aggressiveness, homogeneity, and genetic divergence that make recapitulation of patient tumour characteristics difficult, subcutaneous cell line tumours have the additional drawback of an artificial grafting site that limits vascularisation and tends toward development of stromal fibrosis [218, 219]. Even if an orthotopic grafting site is used, the homogeneity of cell lines lead to the formation of tumours as an unstructured mass of cells lacking the histological features of patient tumours, such as the abnormal glandular structures of clinical PCa [200].

The direct implantation of fresh patient tumour pieces to generate patient-derived xenograft (PDX) models can thus be used to overcome limitations associated with traditional *in vivo* models. Cancer cells are of a human origin, and tumours retain genetic and histological features observed in the clinic. They have been demonstrated to be both useful and superior for replacing cell line tumours in various investigative and experimental applications including therapeutic target discovery, drug efficacy studies, and personalized cancer therapy [220].

1.2.3.1 Generation and Characterization of PDX Models at the Living Tumour Laboratory (LTL)

PDX models at the Living Tumour Laboratory (LTL) are generated using fresh tumour tissue from patients undergoing surgery or biopsy. The subrenal grafting site is routinely used due to its high vascularisation, allowing for better nutrient and oxygen supply and ensuring higher (>90%) take rates compared to alternative sites [218]. In cases of PCa, patient tumour specimens were cut into smaller fragments and implanted into the subrenal capsule of castrated male nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice supplemented with testosterone. Tumour pieces showing robust growth can then be harvested and serially transplanted into subsequent NOD/SCID mice or frozen with dimethyl sulfoxide (DMSO) in liquid nitrogen for maintenance of seeding stock. If significant tumour

growth persists beyond five generations of serial transplantation, the tumour is considered an established LTL tumour line [220]. In like manner, the LTL has established over 45 PCa tumour lines ranging from highly aggressive NEPC and CRPC to difficult-to-grow hormone-naive primary PCa. Additionally, tumour lines from malignancies originating from other organs such lungs [221], ovaries [222, 223], and breast [224] have also been established and successfully employed in many experimental investigations [225-227]. Collectively, the LTL has over 300 distinct PDX models representing a broad range of malignancies and associated clinical subtypes.

The LTL PDX models have been extensively described by a number of macroscopic, histological, and molecular characteristics. In addition to basic information such as patient profile and tissue of origin, the tumour's doubling time, ability to invade local tissue, and propensity for distant metastasis can all combine to give a rough estimate of aggressiveness. Furthermore, sensitivity to pertinent treatment options (such as castration, second-generation antiandrogens, or docetaxel in cases of PCa) can help delineate various tumour lines [226, 228]. Standard histological assessments such as hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) staining is also used to determine cellular morphology, tissue structure, and expression of specific cell markers for categorizing tumour lines into various subtypes [220, 227]. Additionally, tissue microarrays (TMAs) have been constructed from these tumour lines and can be used to assess expression of various proteins of interest. Finally, global profiling approaches such as array comparative genomic hybridization (aCGH), microarray analysis, and next-generation sequencing have been employed to assess the genetic, transcriptomic, and epigenetic profiles of these LTL tumour lines [127, 220]. The combined use of biological information at these different levels can thus be a powerful tool in helping select the best PDX models for experimental use, allowing a closer approximation to the patient population of interest and yielding more clinically relevant results. In particular, the LTL collection of PCa PDXs contain hormone sensitive (LTL-467) and hormone insensitive (LTL-352) models, NEPC (LTL-370) and adenocarcinoma CRPC (LTL-313BR) models, metastatic (LTL-313H) and non-metastatic (LTL-313B) models, and models harbouring various genetic alterations common to PCa patients including TMPRSS2-ERG fusion (LTL-331R), PTEN deletion (LTL-

545), TP53 mutation (LTL-311), and SPINK1 expression (LTL-418) [127, 220, 228, 229]. Together, they represent a broad spectrum of PCa clinical manifestations and cover the major characteristics and phenotypes associated with PCa development and progression.

1.2.3.2 Advantages of PDX Models in Preclinical Studies

The PDX models at the LTL offer a number of advantages in addressing the significant translational gap between *in vivo* models and patients in the clinic. PDX models are first and foremost high-fidelity representations of patient tumours. Their direct derivation from patient specimen allows them to retain many of the original tumour characteristics, including tissue histological architecture, cellular heterogeneity, mutational landscape, and gene expression profiles [200, 220]. This similarity to donor tissue offers greater predictive power in terms of assessing potential therapeutic responses and mechanisms of treatment resistance. Using the LTL PCa PDXs as examples, not only can various models retain histological distinctions between NEPC and adenocarcinoma, they also show differing sensitivities to various therapeutic agents such as bicalutamide, enzalutamide, and docetaxel [220, 228, 229]. Furthermore, mechanisms of resistance arising in the PDXs are reflective of the eventual outcome in patients. The LTL-331 tumor line was derived from a hormone-naive PCa adenocarcinoma (PSA^+/AR^+) and is initially responsive to castration, showing decreased tumour volume and plasma PSA. However, the PDX tumour becomes resistant and recurs after 6-8 months as typical NEPC ($SYP^+/AR^-/PSA^-$). Importantly, this predicted NEPC transdifferentiation was confirmed in the original patient, who presented with recurrent NEPC 5 years following initial diagnosis [127]. As such, the use of PDX models in a variety of research activities, including preclinical drug efficacy studies in anticancer therapeutics development [181, 229-232], discovery and validation of potential biomarkers or therapeutic targets [16, 223, 233, 234], and personalized cancer therapy can yield highly clinically relevant results superior to conventional animal models.

The clinical relevance of PDXs can be further enhanced by using multiple models together as a panel for drug efficacy evaluations. It is increasingly recognized that over-reliance on a single model

system to reflect patient population responses to therapy is a major deficiency in current drug development approaches [235, 236]. In particular, only a subpopulation of patients in a given clinical setting responds to a given therapy, as evident from objective response rates observed in various clinical trials (e.g., 17% for docetaxel [237], 59% for enzaluatmide [129]). As such, the use of multiple models covering a range of molecular characteristics common to patient tumours can achieve better preclinical modeling of patient populations. Over 45 PCa PDX models are available at the LTL, of which more than 11 can be considered advanced PCa (NEPC or CRPC). Experimentation on an expanded panel using multiple tumour lines could thus take into account potential underlying heterogeneous disease characteristics and offer valuable information on likely-responder populations, helping develop more accurate patient selection criteria for clinical trials.

Finally, PDX models can help lessen a key drawback common to all xenograft models of cancer. The immune system has been widely recognized in recent years to play an important role in cancer development and anticancer therapeutic response [238-240]. This has become especially prominent given the recent clinical successes of therapeutics targeting immune checkpoint molecules such as CTLA-4 and PD-L1 [241, 242]. Unfortunately, uptake and growth of human cancer cells in foreign hosts require the use of immunodeficient animals. Conventional wisdom suggests that such model systems cannot be used to assess functional changes to cancer-associated immunity, and neither can they be used to determine the relative contributions of reactivated anticancer immune response to therapeutic efficacy. While murine cells eventually overtake the stromal compartment of established PDX tumour lines [243, 244], PDX grafts in the early first-generation stage still retain original human stroma, including fibroblast, endothelial cells, and functional tumour-associated immune cells. As such, although first-generation PDXs do not recapitulate complex patient immune-cancer cell interactions in a fully native context, certain gross changes pertaining to cancer immunity can still be observed and experimentally characterized. Analysis of patient immune cell populations in first-generation PDXs could therefore uniquely supplement traditional efficacy data and potentially reveal enhanced therapeutic efficacy from synergistic reactivation of anticancer immunity.

1.3 Altered Cancer Metabolism

Modern approaches to understanding cancer biology have been aided by the classification of various processes into demarcated characteristics known as cancer hallmarks. The contributions of various genes and gene products, together with abnormal functions and pathway deregulations arising from mutations, have been described extensively in their ability to assist cancer initiation and progression via these core characteristics. While the current list of ten hallmarks is comprehensive in documenting cancer cell behaviour and includes abilities such as resisting cell death, inducing angiogenesis, and evading immune destruction [245], recent discoveries seem to indicate that a further expanded list of characteristics may become necessary in the near future. There is mounting evidence that epigenetic mechanisms such as histone modifications and non-coding RNAs [246-248], autophagy [249, 250], and the tumour stromal compartment [243, 251-253] can all play critical roles in regulating cancer development and response to therapy. However, as the complexity of cancer biology becomes increasingly apparent, it is not at all clear whether various established and potential cancer hallmarks can be considered equally essential. More specifically, certain hallmarks, such as resisting cell death and evading immune destruction, seem foundational as they contribute directly to cancer survival. Other hallmarks, such as tumour-promoting inflammation and activating invasion, seem more peripheral and contribute instead to aspects of cancer aggressiveness. As such, the therapeutic targeting of fundamental yet cancer-specific characteristics could theoretically yield more effective treatment options applicable to many cancer types.

Deregulated cellular energetics via altered cancer metabolism is one such hallmark that pertains to the essential energy-gathering ability of cancer cells for survival and growth. Aberrations to multiple metabolic pathways involving each of the major subclasses of biological macromolecules have been implicated in various cancer contexts. For example, glioma cells have been found to exhibit MYC-driven glutamine addiction, resulting in elevated glutamine uptake beyond the nitrogen requirements for protein and nucleic acid biosynthesis [254-256]. Changes to lipid metabolism and fatty acid biosynthesis have also been observed in prostate, breast and colorectal cancer. The overexpression of SREBPs and FASN

stimulates *de novo* fatty acid synthesis from glycolytic substrates and fuels membrane production and lipid-based post-translational modifications [257-259]. The redirection of glucose into the pentose phosphate pathway has also been observed in a number of cancers, providing precursors to nucleic acid biosynthesis and supplying abundant NADPH to counteract oxidative stress [260]. This is facilitated by hyperactivation of key pathway enzymes such as G6PDH via oncogenic signals downstream of PI3K/AKT, KRAS, or SRC [261, 262]. Finally, the production of novel oncometabolites due to enzymatic mutations can also be categorized under reprogrammed cancer metabolism. Mutations at arginine-132 in IDH1 or the analogous arginine-172 in IDH2 can be found in up to 70% of advanced glioma patients [263, 264]. This mutation leads to an acquired ability to produce 2-hydroxyglutarate from α-ketoglutarate [265]. In turn, 2-hydroxyglutarate acts as a competitive inhibitor of other α-ketoglutarate-dependent enzymes, the most significant being histone demethylases and 5-methlycytosine hydroxylases. This results in global genome-wide alterations to histone and DNA methylation patterns, affecting gene expression and contributing to glioma initiation and progression [266].

Generally, altered cancer metabolism manifests clinically as aberrant increased uptake of various metabolites. This unique phenomenon has been particularly applicable to cancer detection and imaging. In many cases, a positron-emitting radiolabel such as carbon-11 or fluorine-18 is incorporated into inactive metabolic analogues and administered to patients for PET imaging. The elevated metabolic requirements of cancer cells result in an increased tracer uptake compared to normal tissue, while the tracer's metabolic inactivity further contributes to its selective accumulation within the tumour. A number of metabolites involved in lipid and amino acid metabolism, including acetate [267, 268], choline [269, 270], glutamine [271], methionine [272, 273], and their respective derivatives have been used in clinical and investigational settings for detecting cancer and measuring therapeutic response [274]. Furthermore, strategies to pharmacologically inhibit these altered metabolic pathways are being actively investigated as promising avenues for developing effective treatment options [256, 275-277].

1.3.1 Cancer-generated Lactic Acid and the Acidic Tumour Microenvironment

Beyond documented changes to glutamine metabolism, fatty acid synthesis, and production of oncometabolites, the most recognized and widespread reprogrammed metabolic pathway in cancer is altered glucose metabolism in the form of elevated aerobic glycolysis. Under normal physiological conditions, glucose is fully catabolised into CO_2 via glycolysis and the TCA cycle. Glycolytic enzymes break down one molecule of glucose into two molecules of pyruvate, which then enters the TCA cycle to generate three molecules of CO_2 and four molecules of NADH per pyruvate molecule. The NADH is in turn oxidized in the mitochondria via the electron transport chain for the production of ATP in a process known as oxidative phosphorylation. This is considered the primary mechanism by which normal cells obtain energy, with each molecule of glucose ultimately providing between 30 to 36 molecules of ATP. However, as oxidative phosphorylation is highly dependent upon oxygen availability, the utilization of pyruvate can be diverted away from the TCA cycle to produce lactic acid during instances of low oxygen, such as during periods of strenuous exercise [278]. This anaerobic glycolysis ensures the regeneration of NAD^+ and continued production of ATP, albeit at much lower amounts [279].

Cancer cells, however, have long been known to divert pyruvate towards lactic acid production for energy needs even under conditions of abundant oxygen. This propensity towards aerobic glycolysis was first observed by Otto Warburg as early as the 1920s [280] and has been termed the Warburg effect. As this redirected metabolism is much less energetically efficient than oxidative phosphorylation, an accompanied increase in glucose consumption is often observed [281, 282]. This elevated glucose uptake is commonly exploited in clinical practice by FDG-PET imaging of many major malignancies including breast, colorectal, head and neck, lung, and ovarian cancer [274, 283, 284]. Increased glycolytic flux through aerobic glycolysis also leads to an overproduction of downstream lactic acid, which is ultimately eliminated from the cancer cell by secretion into the tumour microenvironment. This elevated presence of cancer-generated lactic acid results in the local acidification of the tumour and its surroundings, which is another phenomenon commonly observed in cancer. Intratumoural pH frequently drops from a physiological pH of 7.4 to an acidic tumour microenvironmental pH of 6.0 to 6.5 [285, 286].

Furthermore, in addition to elevated glycolysis, excessive lactic acid production can also result from increased glutaminolysis. This is facilitated by anaplerotic reactions that replenish TCA cycle intermediates, converting glutamine to α-ketoglutarate, which enters the TCA cycle and is converted into malate. Malic enzyme can then convert malate back into pyruvate for lactate production [254, 287, 288]. Although lactic acid is conventionally considered an inconvenient "waste" product requiring detrimental adaptations from cancer cells to ensure continued growth and survival [289], there is increasing evidence that lactate and the resultant acidic tumour microenvironment plays an active and crucial role in fuelling various fundamental aspects of cancer development and progression.

1.3.2 Benefits to Proliferation, Angiogenesis, and Metastasis

Many have speculated on the purpose of altered cancer metabolism, particularly of aerobic glycolysis, since it was first observed. Otto Warburg himself suggested that mitochondrial defects impairing normal oxidative phosphorylation in cancer cells might necessitate such metabolic changes [290]. Further studies, however, have since demonstrated that only certain cancers harbour critical mitochondrial defects making aerobic glycolysis necessary [291], thus only partially accounting for this near-universal phenomenon. The majority of cancer cells are able to revert back to oxidative phosphorylation should the generation of lactic acid become inhibited [292, 293]. This suggests that altered cancer metabolism in the form of elevated aerobic glycolysis has purposes beyond overcoming the inability to derive ATP from oxidative phosphorylation.

As elevated aerobic glycolysis remains perplexing from an energetics perspective, generating ATP approximately 18-fold less efficiently than oxidative phosphorylation, current understanding have focused on the broader benefits to proliferation that altered metabolism may confer [289, 294, 295]. The predominant theory suggests that proliferative advantage comes from both an incomplete catabolism of glucose and a resistance towards hypoxic conditions. By not fully metabolising glucose into CO_2 through the TCA cycle, upstream intermediates in the glycolysis pathway can be redirected as precursors for nucleotide and lipid production, thus generating sufficient building blocks for rapid synthesis of various

cellular components and biomacromolecules [296, 297]. Furthermore, as tumours frequently experience fluctuating levels of oxygen, alternating between normoxic and hypoxic conditions at inconsistent intervals due to abnormal vascularizations [298, 299], a reliance on oxygen-independent glycolysis could allow sustained proliferation and decrease susceptibility to hypoxic stress during spontaneous periods of low oxygen [289]. An extension to this theory has been recently proposed and includes a symbiotic relationship between hypoxic and normoxic cancer cells. Under this "lactate shuttle" hypothesis, glucose is primarily utilized by hypoxic cancer cells and secreted as lactic acid following anaerobic glycolysis. The lactate is then shuttled to normoxic cancer or tumour stromal cells and converted back to pyruvate to fuel oxidative phosphorylation [279, 300, 301]. This theoretically facilitates maximal proliferation regardless of the experienced tumour microenvironmental conditions. However, sustained proliferation is not the sole hallmark characteristic of cancer, and a focused view on the proliferation benefits of altered cancer metabolism seems unnecessarily restrictive.

Involvement of aerobic glycolysis in support of other hallmark cancer characteristics is also well documented. One such aspect of cancer development is the ability to induce new blood vessel formation for improved nutrient supply. Sprouting angiogenesis, whereby new capillaries are formed from existing vessels, is the form of angiogenesis most closely associated with tumour growth. This process is facilitated through VEGFR signaling in endothelial cells. Upon VEGF binding and receptor activation, endothelial cells secrete proteases to degrade the local basement membrane and extracellular matrix. This allows them to migrate into the surrounding extracellular space and proliferate, creating a budding lumen as the beginnings of an immature blood vessel [302, 303]. Under normal physiological conditions, a number of additional ligands and cell types are involved in the structural and functional maturation of nascent vessels. However, these factors are often inappropriately regulated in the tumour, disrupting proper vessel formation and resulting in abnormal vasculature networks [304]. Although accumulation of lactate in damaged tissues trigger angiogenesis as a natural part of the wound healing process [305], it can be co-opted by a lactate-rich acidic tumour microenvironment to induce blood vessel formation in the tumour. Importantly, multiple studies have demonstrated that lactic acid is indeed a driving force

contributing to an angiogenic phenotype [306]. The accumulation of lactate can protect NDRG3 from VHL-mediated degradation, allowing it to activate the Raf/ERK pathway to promote angiogenesis [307]. Extracellular lactate can also stimulate endothelial cell tubulogenesis for vessel sprouting via the PI3K/AKT pathway and signaling cascades of multiple receptor tyrosine kinases [308]. Furthermore, endothelial cells can import lactic acid from the environment using lactate transporters, activating HIF-1α and stimulating the NFκB/IL-8 pathway for endothelial cell migration and tube formation [309, 310]. Finally, VEGF production can be triggered in macrophages and endothelial cells by excessive lactic acid, thus inducing endothelial cell migration and initiating in the angiogenic process [311].

Local tissue invasion and distant organ metastasis can also be facilitated by excessive lactic acid production and subsequent acidification of the tumour microenvironment. In particular, increased lactate can stimulate degradation of the extracellular matrix and induce EMT to drive invasion into tumour-adjacent normal tissue. Tumour-surrounding tissues subjected to local acidosis as the acidic milieu diffused outward were found to be more susceptible to cancer invasion [312]. This acid-mediated process results from both the death of surrounding normal cells and the secretion and activation of numerous proteases responsible for extracellular matrix degradation and remodelling, including MMPs, cathepsins, and collagenases [313-315]. Furthermore, proteins and transporters associated with acid-generating pathways have been shown to localize to the leading edge of tumour invasion and enhance cell motility. On the tumour level, expression of the glucose transporter GLUT1 and the proton pump NHE1 were found to be upregulated in cells on the outer invasive edge [316, 317]. From a cellular level, lactate transporters such as MCT1 and MCT4 are localized to the leading edge of lamellapodia of migrating cells, for which inhibition abrogated invasive and migratory potentials [318-320]. Various glycolytic enzymes associated with elevated aerobic glycolysis can also induce an EMT phenotype in cancer cells, reducing E-cadherin expression, increasing SNAIL expression, and promoting cytoskeleton remodeling for increased cell motility [321-323]. Finally, the induction of angiogenesis as previously described can also provide systemic access for migratory cancer cells and help seed distant organ metastasis. As such, the acidic tumour microenvironment plays an important contributing role to cancer invasion and

metastasis by creating a toxic environment to adjacent normal cells, degrading the extracellular matrix with activated proteases, increasing cancer cell motility, and promoting angiogenesis.

1.3.3 Immunosuppressive Effects of Cancer-generated Lactic Acid

While the involvement of cancer-generated lactic acid in the established hallmarks of proliferation, angiogenesis, and metastasis is well-studied, its contribution to the emerging hallmark of avoiding immune destruction is still relatively underappreciated despite its equal, if not potentially greater, importance in fundamentally supporting tumour survival, growth, and progression. Our current understanding of the immune system's role in cancer development revolves around the concepts of immunosurveillance and immunoediting [238, 324]. During the early phase of cancer initiation, the emergence of transformed cells releases danger signals into the surrounding environment [325], either as degraded extracellular matrix products resulting from angiogenesis and local tissue invasion, or as immunogenic mutated epitopes presented on MHC molecules [326]. These signals are recognized by the immune system and trigger an anticancer immune response via cytotoxic immune cell-mediated induction of cancer cell death. In an ideal situation, this process of immunosurveillance fully eliminates the initiating cancer cells and offers protection. However, in scenarios where some cancer cells remain after initial elimination, the process of immunoediting begins. Residual cancer cells enter an equilibrium phase with the immune system, where they continue to proliferate and accumulate mutations. Some of these alterations are detected and eliminated by the immune system, while other aberrations remain immunologically silent and continue to escape immune detection. Eventually, this extended period of immune sculpting selects for cancer cells that have acquired potent mechanisms of avoiding immune destruction, ultimately leading to an outgrowth of cancer cells that have fully escaped immunosurveillance and are clinically detectable [238, 324].

The immune system as it relates to cancer can be broadly divided into an effector arm responsible for eliciting anticancer immunity and a regulatory arm responsible for suppressing immune action. Many mechanisms tipping the balance in favour of immunosuppression, either through inhibiting the effector

arm or enhancing the regulatory arm, have been implicated in cancer's ability to actively avoid immune destruction. For example, the infiltration of regulatory immune cells such as regulatory T cells, type II (M2) macrophages, and myeloid-derived suppressor cells (MDSCs) are commonly associated with poor prognosis in many cancers [327-329]. Conversely, the presence of effector cell types such as cytotoxic $CD8^+$ T cells and natural killer (NK) cells offer better prognosis [330-332]. An immunosuppressive tumour microenvironment can also be perpetuated by the production of regulatory cytokines such as TGFβ and IL-10, attenuating anticancer immunity by inhibiting production of pro-inflammatory cytokines such as IFNγ, hampering cytotoxic T cell functions, and skewing differentiation of recruited immune cells towards the immunosuppressive phenotypes [333-335]. Anticancer immunity can be further inhibited by cancer cells through the expression of checkpoint receptor ligands such as CD80 and PD-L1 on the cell surface, thereby directly transmitting inhibitory signals to prevent T cell co-activation and induce T cell cycle arrest [241, 336, 337]. Finally, amino acid metabolizing enzymes can also reduce anticancer immune functions within the tumour. Two such enzymes, IDO and ARG1, are commonly overexpressed by both cancer cells and tumour-associated suppressor cell types such as M2 macrophages, MDSCs, and plasmacytoid dendritic cells. The depletion of arginine into ornithine and urea by ARG1 results in T cell anergy from loss of TCR expression and T cell cycle arrest at the G_0/G_1 phase from decreased cyclin D3 and CDK4 [338, 339]. The catabolism of tryptophan into kynurenines by IDO also results in an inhibition of T cell proliferation and reduction of TCR expression, while the resultant overabundance of kynurenines further skews the conversion of effector T cells into regulatory T cells [340, 341].

However, one additional mechanism that can significantly assist cancer cells in avoiding immune destruction is the largely ignored contributions of the acidic tumour microenvironment. There is growing evidence that increased extracellular acidity as caused by excessive lactic acid production can greatly hamper the anitcancer functions of multiple immune cell types [342, 343]. Lactic acid can induce anergy in cytotoxic T lymphocytes by suppressing proliferation, inhibiting cytokine production, impairing T-cell recognition of presented tumour antigens, reducing TCR expression, and attenuating cytotoxic activity

[344-348]. Similarly, the cytotoxicity and IFNγ-producing ability of anticancer NK cells can be reduced by cancer-generated lactic acid via decreased perforin and granzyme expression and inhibition of NFAT upregulation [349, 350]. With regard to immune cells in the regulatory arm, the presence of lactic acid can suppress monocyte differentiation and impair the antigen-presentating abilities of dendritic cells [351]. The differentiation of tumour-associated macrophages is also skewed in the presence of lactic acid, favouring the suppressive M2 phenotype and inducing VEGF and ARG1 expression [352, 353]. A greater presence of MDSCs in lactate-rich tumours has also been observed, resulting in the increased suppression of NK cell activity. However, the exact mechanism of their lactate-mediated generation and recruitment remain to be further elucidated [350].

Considering the crucial role that avoiding immune destruction plays in ensuring cancer survival and progression, and taking into account the near-universal propensity of solid tumours to adopt altered metabolism in the form of elevated aerobic glycolysis, we proposed that the resulting acidification of the tumour microenvironment from excessive lactic acid production plays a fundamental but oft-neglected role in maintaining tumour-localized immunosuppression [200, 343]. The central immunosuppressive role of cancer-generated lactic acid tips the immune balance away from anticancer immunity through direct action on multiple immune cell types, impairing the cytotoxic and pro-inflammatory abilities of anticancer effector cells while promoting the differentiation and function of regulatory immune cells in the tumour microenvironment.

1.3.4 Summary of Novel Hypothesis

In addition to effects on proliferation, angiogenesis, metastasis, and immunosuppression, the acidic tumour microenvironment can further modulate cancer growth by causing global epigenetic alterations [354-356], affecting treatment efficacy of weak acid/base therapeutics [357, 358], and increasing autophagic flux [359, 360]. However, given the fundamental nature of cancer hallmark characteristics in governing cancer survival and progression, the primary purpose of an altered cancer metabolism in creating a lactate-mediated acidic tumour microenvironment is likely to promote these core

cancer abilities. As such, advantages to proliferation, angiogenesis, tissue invasion/metastasis, and especially to evading immunosurveillance can be considered the predominant downstream effects of an altered cancer metabolism.

We have previously summarized earlier findings in the literature and have proposed that cancer-generated lactic acid and an acidic tumour microenvironment play a critical role in facilitating cancer growth and development. A particular emphasis was placed on the largely overlooked ability of such altered cancer metabolism to create a local immunosuppressive tumour microenvironment (Figure 1.1) [200, 343]. From a therapeutic perspective, our novel hypothesis that lactic acid is a multi-functional metabolite suggests that there is a previously underappreciated interrelatedness between seemingly diverse cancer characteristics. As such, a therapeutic strategy that inhibits the secretion of cancer-generated lactic acid could theoretically achieve improved synergistic efficacy as though multiple hallmarks were combinatorially targeted simultaneously.

1.3.4.1 The Monocarboxylate Transporter (MCT) Family as Therapeutic Targets

Given that lactic acid can come from a diverse range of upstream metabolite sources including glucose, glutamine, and their related metabolic intermediates, the ability for cancer cells to excrete lactic acid into the tumour microenvironment could prove to be a critical junction for effective therapeutic intervention. Cellular lactate transport is primarily facilitated by a family of twelve-pass membrane channel proteins known as monocarboxylate transporters (MCTs). These MCTs catalyze the proton-linked transfer of various metabolic monocarboxylates, including lactate, pyruvate, acetate, ketone bodies, and amino acids, showing different affinities depending on the metabolite and the MCT isoform [361]. The ancillary protein CD147 is also required for their proper membrane localization and function [362]. MCT1 (SLC16A1) and MCT4 (SLC16A3) are the most widely distributed and best studied, with MCT1 ubiquitously expressed in almost all tissues and MCT4 expression more restricted to highly glycolytic tissue types such as skeletal muscles, astrocytes, and mammalian cell lines [363]. MCT2 (SLC16A7) expression appears predominantly in neuronal tissues [364] and MCT3 (SLC16A8) expression is found

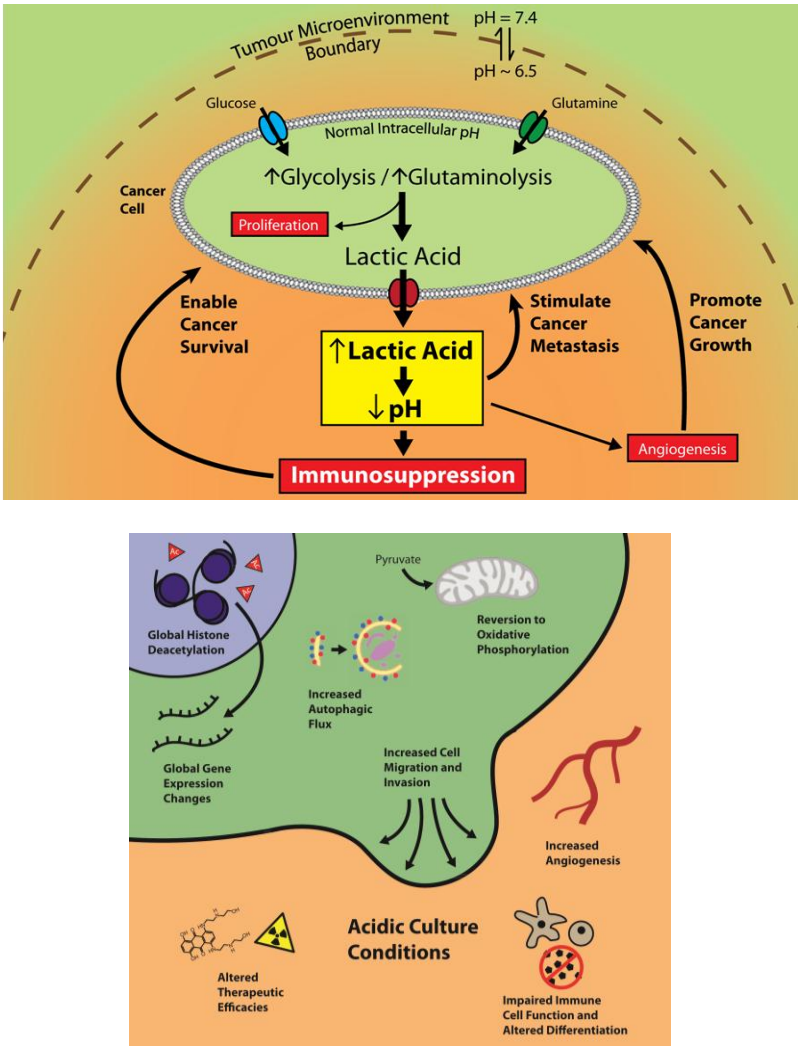

Figure 1.1. Two previously published graphical summaries of our novel hypothesis detailing the proposed model for a central, cancer-promoting and immunosuppressive role of lactic acid as generated and secreted by cancer cells. We propose that excessive cancer-generated lactic acid resulting from altered energy metabolism leads to a decreased pH in the tumour microenvironment, which in turn promotes multiple oncogenic processes including proliferation, tissue invasion/metastasis, angiogenesis, and suppression of the local anticancer immune response [343]. An acidic tumour microenvironment can also modulate additional cancer-associated properties, inducing global epigenetic changes, increasing autophgic flux, and altering therapeutic efficacies [200]. As such, inhibition of lactic acid production and subsequent reversal of the multiple downstream cancer-promoting processes could result in a synergistically effective therapeutic approach.

almost exclusively in the retinal pigment epithelium [365]. MCT8 (SLC16A2) and MCT10 (SLC16A10) are responsible for the transport of thyroid hormones and other aromatic amino acids [366]. The remaining MCT family members remain largely uncharacterized [367, 368].

MCT1 and MCT4 have been widely reported to play an important role in facilitating development and progression of multiple cancer types. Their overexpression is associated with poor prognosis in breast [369], head and neck [370], renal [371, 372], bladder [373], pancreatic [374], and non-small cell lung cancer [375]. Furthermore, they have been functionally demonstrated to significantly contribute to cancer cell proliferation [376, 377], resistance to hypoxia [378], induction of angiogenesis [309, 310], and stimulation of invasion/metastasis [379]. More specifically, the overexpression of MCT4 is also associated with poor prognosis in PCa, correlating to higher PSA levels and Gleason scores, earlier times to recurrence, and progression to treatment resistance [380-382]. As such, the therapeutic inhibition of MCT function can be an effective treatment strategy for the clinical management of multiple cancer types, including PCa. Given the near-ubiquitous expression of MCT1 in normal tissues, and in particular its high expression in key organs such as lungs, kidneys, heart, and liver, the targeting of more cancer-specific MCT isoforms may offer a safer approach for therapeutic intervention. MCT4 could thus be a better candidate for inhibiting lactic acid export in a cancer context, balancing a potentially reduced toxicity to normal tissues while still remaining relevant to altered cancer metabolism in a wide spectrum of cancer types.

1.4 Therapeutic Agents

1.4.1 Antisense Oligonucleotides (ASOs)

Antisense oligonucleotides (ASOs) form a class of therapeutic compounds containing synthetic nucleotide sequences of 16 to 22 bases in length. These sequences are designed to be complementary to a target transcript sequence via traditional Watson-Crick base pairings and have backbone modifications for improved pharmacokinetic profiles. More specifically, the non-bridging phosphoryl oxygen of DNA is replaced with sulphur to create a first-generation phosphorothioate (PS) backbone more resistant to serum

nuclease degradation [383, 384]. Further chemical modifications surrounding the 2’ carbon in the sugar moiety have yielded second-generation ASOs with 2'-O-methoxyethyl (2MOE) groups, giving improved tissue distribution and week-long elimination half-lives [385]. Current nucleotide chemistry employed in clinical trials of various ASOs involve generation 2.5 modifications, using 2’-4’ constrained ethyl (cEt) modified bicyclic nucleotides to flank a central DNA gap. This results in an improved efficacy despite comparable tissue distribution [386, 387] and allows for a reduction in ASO length to reduce manufacturing costs [388].

Mechanistically, the binding of ASOs to their target mRNA results in RNase H-mediated transcript degradation [389]. Furthermore, the DNA:RNA duplex can also sterically hinder ribosomal readthrough during protein translation [390] and prevent proper RNA splicing events leading to nonsense-mediated mRNA decay [391]. Ultimately, the effective binding of ASOs to target mRNA sequences result in the inhibition of target protein expression. Theoretically, the use of ASOs allow for a quick identification of selective and specific inhibitory therapeutic compounds based simply on a known mRNA sequence, achieving functionally equivalent treatment effects while bypassing traditional chemical screens and target specificity verifications. Furthermore, the alternate mechanism in which ASOs modify gene splicing could open therapeutic avenues not amenable to small molecule targeting. However, practical hurdles associated with tissue distribution and *in vivo* target knockdown remain to be overcome [392]. Recent FDA approval of the ASOs mipomersen, which inhibits the synthesis of apolipoprotein B for treatment of homozygous familial hypercholesterolemia [393], and nusinersen, which modulates SMN2 mRNA splicing for treatment of spinal muscular atrophy [394], together with the FDA fast-track designation of alicaforsen, an ICAM-1 ASO for treatment of ulcerative colitis [395], suggests that ASOs remain a viable therapeutic strategy and could still be useful in the clinic. A number of ongoing clinical trials targeting overexpressed genes commonly found in cancers, such as STAT3 [396, 397], AR [398], HSP27 [399, 400], and KRAS [401] have shown promising preliminary results.

1.4.2 Small Molecule Inhibitors (SMIs)

Small molecule inhibitors (SMIs) comprise by far the largest category of therapeutic agents used in clinical settings. In the cancer context, the discovery of novel therapeutic chemicals have transitioned away from cytotoxic molecules that broadly inhibit cell proliferation to more selective targeted inhibitors of various oncogenic drivers of cancer growth and progression [402]. Classic examples include hormonal analogues of estrogen and testosterone for the treatment of breast and prostate cancer, as well as kinase inhibitors against BCR-ABL in chronic myeloid leukaemia, EGFR in non-small cell lung cancer, and BRAF in melanoma [403, 404]. The advent of genome-wide approaches to characterizing malignancies has further accelerated our understanding of cancer biology, revealing novel drivers of progression and mechanisms of treatment resistance. Ideally, such technology can lead to the application of truly personalized cancer therapy in the clinic, closely matching patients to effective inhibitors based on information from individualized tumour profiling [405]. However, the severe lack of effective therapeutic compounds remains the biggest translational gap preventing a tailored patient-specific approach to disease management. Many identified recurrent mutations still remain clinically inactionable, and many well-established cancer-associated gene products are still considered "undruggable" [406, 407]. This problem is further compounded by the high attrition rate throughout the many intermediary steps along an extended drug development process, from target validation, chemical hit identification, lead optimization, to ultimately clinical trials in patients [402]. As such, the identification of efficacious SMIs targeting multiple fundamental aspects of cancer biology is still urgently needed for clinical use.

Inhibition of altered cancer metabolic pathways without affecting normal cell metabolism remains an active area of cancer therapeutics research [294]. As the lactic acid transport activity of MCTs play a key role in facilitating and maintaining elevated aerobic glycolysis, a number of potential SMIs have been reported in the literature. Early biochemical studies elucidating the substrate affinities and functional differences between various MCTs have used substituted aromatic monocarboxylates such as CHC, sulfhydryl reagents such as pCMBS, polyphenols such as phloretin and quercetin, and anion transport inhibitors such as DIDS as competitive inhibitors of MCT transport activity [363, 408-410].

However, these initial compounds suffered from low affinity and lacked isoform specificity, broadly inhibiting all studied MCTs [411]. Lipophilic statins such as fluvastatin and atorvastatin have also been shown to have MCT4-inhibitory activities absent from hydrophilic statins such as rosuvastatin and pravastatin. However, their clinical application in the altered cancer metabolism context is limited as lipophilic statins are known to have cytotoxic effects, and concentrations required for MCT4 inhibition are multiple-folds higher than therapeutically relevant plasma concentrations [412]. More recent attempts to develop MCT-specific inhibitors using high-throughput screening strategies have yielded AZD3965, an inhibitor with nanomolar affinities specific to MCT1 and MCT2. Although it was originally developed as an immunosuppressant inhibiting T-cell proliferation [413], it is currently in phase I clinical trial with potential efficacy against small cell lung cancer and lymphomas [414, 415]. Other reported compounds in preclinical stages of development include londamide, which has greater affinity towards mitochondrial pyruvate carrier [416], and 7ACC derivatives, which inhibit lactate import via MCT4 but not export [417]. As such, there is still a lack of effective SMIs that can specifically inhibit lactic acid secretion from cancer cells via MCT4.

1.5 Overall Working Hypothesis

Given that altered cancer metabolism in the form of elevated glycolysis plays a fundamental role in promoting multiple oncogenic processes including proliferation, angiogenesis, invasion/metastasis, and suppression of anticancer immunity, and that MCT4 seem to facilitate these processes in a cancer-specific context by secreting lactic acid into the tumour microenvironment, we propose that blocking lactic acid transport via MCT4 will result in a combination of direct cellular effects and indirect tumour microenvironmental effects that lead to enhanced cancer cell death. In particular, inhibition of excessive lactic acid secretion due to elevated glycolysis will induce intracellular acidification and hamper metabolism in cancer cells, reducing cell proliferation. Furthermore, the reduction in lactic acid secretion will restore the tumour microenvironmental pH to physiological levels, thus inhibiting the tissue invasive, angiogenic, and immunosuppressive effects of an acidic tumour microenvironment to further stunt tumour

growth (Figure 1.2). As such, by blocking the secretion of cancer-generated lactic acid and alleviating its effect on driving multiple fundamental cancer-associated downstream processes, we could theoretically achieve synergistically improved treatment efficacy even by single-agent targeting as though multiple hallmarks were simultaneously inhibited. Furthermore, we think this therapeutic strategy would be applicable to a broad spectrum of glycolytic solid tumours beyond advanced PCa.

The overall working hypothesis will be investigated through the following specific aims: *Specific Aim 1:* To determine the clinical relevance of elevated glycolysis in advanced PCa

Specific Aim 2: To determine the effectiveness of MCT4 inhibition at reducing advanced PCa growth and reversing various lactic acid-associated downstream processes in a proof-of-concept study.

Specific Aim 3: To begin the process of developing MCT4 SMIs for clinical use.

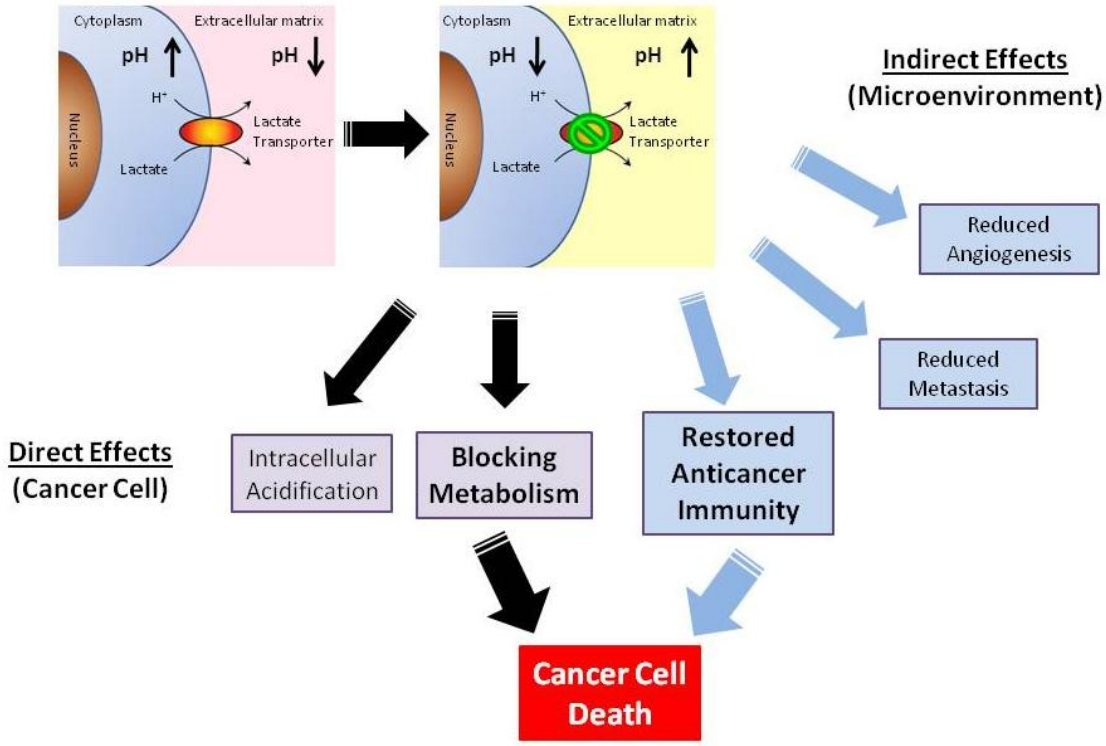

Figure 1.2. Our hypothesis that MCT4 inhibition will be an effective therapeutic strategy given the critical role lactic acid plays in facilitating multiple fundamental aspects of cancer biology. As MCT4 is considered the major membrane transporter mediating lactic acid secretion by cancer cells, inhibition of MCT4 will prevent lactic acid efflux. This will lead to 1) direct inhibitory effects on cancer cells via intracellular acidification from accumulated lactate and blockage of altered cancer metabolism. Furthermore, inhibition of MCT4 will also lead to 2) indirect inhibitory effects on tumour growth via the reduction of extracellular lactate levels and restoration of physiological extracellular pH in the tumor microenvironment. This will result in reduced angiogenesis and tissue invasion/metastasis, and more importantly, restored patient anticancer immunity. As such, inhibiting the MCT4-facilitated excessive lactic acid production can synergistically enhance therapeutic efficacy by simultaneously inhibiting cancer cell proliferation, stimulating patient anticancer immune response, and reversing additional downstream tumor-promoting processes associated with an acidic tumour microenvironment as induced by elevated glycolysis. We think this therapeutic strategy will be effective against a broad range of glycolytic malignancies.

Chapter 2: Elevated Glycolysis in Advanced PCa PDX and Patients

2.1 Introduction

Human prostatic fluid is known to contain remarkably high concentrations of citrate, ranging from 40 to 150mM compared to 0.2mM normally found in blood plasma [418]. It is thought that this high concentration of citrate can buffer free calcium ion levels in human semen to regulate sperm functions and maintain healthy sperm activity [419, 420]. In order to maintain a high level of citrate production, human prostate epithelial cells adopt a unique metabolic profile different than the canonical metabolism observed in other normal cells. As citrate is primarily synthesized in the mitochondria from oxaloacetate and acetyl-CoA during the first step of the TCA cycle, a mechanism to replenish both intermediary metabolites and prevent further metabolism of citrate is necessary. While glucose catabolism via glycolysis remains the primary source of acetyl-CoA in normal prostate epithelial cells, oxaloacetate is supplied from an increased consumption of aspartate [421]. The high-affinity aspartate transporter EAAC1 facilitates this intracellular accumulation of aspartate, which is then transaminated by mAAT to form oxaloacetate [422]. In order to inhibit further metabolism of citrate through the TCA cycle, prostate epithelial cells are also known to accumulate high levels of zinc through the ZIP1 transporter [423]. Zinc is an inhibitor of mitochondrial aconitase, thus preventing the conversion of citrate to isocitrate for downstream TCA reactions [424]. During malignant transformation, the prostate's ability to accumulate zinc and produce citrate is significantly reduced [425]. This phenomenon is well-documented in the clinic and has been suggested as a diagnostic marker for clinical PCa detection [426]. For example, non-invasive *in vitro* measurements of citrate concentration in seminal fluid has been shown to have better performance at detecting PCa than traditional serum PSA tests [427, 428], while *in situ* proton magnetic resonance spectroscopic imaging can identify regions of low citrate and high choline for the detection and localization of cancer within the prostate [429-431].

In addition to reduced citrate production, another aspect of altered metabolism pertinent to PCa is the intriguing exception that, unlike most other cancer types, PCa in primary treatment-naive cases are generally not known to exhibit the Warburg effect to a great extent. Traditional FDG-PET is not

considered a reliable imaging technique for staging and diagnosing PCa [432], leading most researchers and clinicians to dismiss elevated glycolysis as irrelevant. However, a number of factors contribute to the difficulty in utilizing FDG-PET in the clinical PCa context, including high false positivity from prostatic inflammation and postoperative scar tissues [433, 434], close proximity of the urinary bladder [435], differences between aggressive and indolent disease [436, 437], and true metabolic heterogeneity in utilizing alternate fuel sources [438, 439]. Nevertheless, there is evidence from clinical FDG-PET studies that suggest elevated glycolysis may be more relevant to late-stage aggressive PCa. For example, intraprostatic FDG uptake were observed more frequently in high-grade PCa and was correlated with a lower 5-year cancer-free survival probability [440], while a majority of PCa metastatic lesions also show FDG-PET positivity [441]. Furthermore, it has been recently demonstrated that NEPC tumours can also be FDG-PET positive, thus potentially providing a useful tool to clinically monitor NEPC tumour viability following treatment [442]. Taken together, elevated glycolysis and a subsequent overproduction of lactic acid may not be entirely irrelevant to clinical PCa. In particular, in line with our hypothesis, an acidic tumour microenvironment may pertain especially to more aggressive subtypes of PCa needing to facilitate a greater number of downstream lactate-associated cancer characteristics. Given that the metabolic phenotype of these aggressive PCa remain poorly characterized, a preliminary description of the predominant metabolic pathways commonly utilized by advanced PCa, particularly of recurrent treatment-resistant CRPC and NEPC, can help guide future therapeutic development and offer early evidence to support our hypothesis regarding the central role of cancer-generated lactic acid in promoting cancer growth and progression.

Recent advances in “omic” technologies have provided the tools to comprehensively describe multiple aspects of biology in great depths of detail. However, the challenge still remains regarding how to best interpret the tremendous amounts of available computational data to glean biologically relevant insights [443, 444]. We too have previously created an extensive in-house database describing our collection of PCa PDX models using genomic and transciptomic approaches, a portion of which have been published and made available through the LTL website [220]. Nevertheless, much about the

phenotypic characteristics of our PDX models as detailed by these methodologies and the clinical scenarios they represent are still to be uncovered and fully appreciated. More specifically, the metabolic profile of our PCa PDXs and their ability to reflect the metabolic phenotypes of clinical patient PCa remain to be assessed. As such, we focused on maximizing the value of existing in-house and publically available datasets, using readily available transcriptomic information to estimate alterations to tumour metabolism during progression from primary treatment-naive PCa to the more advanced treatment-resistant PCa. Since there are enough reasons to believe that a lactate-induced acidic tumour microenvironment can stimulate multiple cancer-promoting processes, we hypothesize that the contributions of elevated glycolysis and excessive lactic acid production is relevant to the more aggressive CRPC and NEPC.

2.2 Materials and Methods

All materials and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated.

2.2.1 Generation and Selection of PDX Models

Patient samples were obtained after informed consent following the protocol approved by the Clinical Research Ethics Board at the University of British Columbia (UBC) and the British Columbia Cancer Agency (BCCA). Animal care and experimental procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care (CCAC). Detailed experimental techniques on the establishment and use of serially transplantable PDX models have been extensively described elsewhere [218, 220]. In brief, fresh PCa tissues from patients undergoing biopsy or radical prostatectomy were grafted under the kidney capsule of 6-to 8-weeks-old male NOD/SCID mice supplemented with testosterone. Serial passaging of viable and rapidly growing tumours were done by harvesting the original tumour tissues, dividing them into smaller pieces, and transplanting them into the subrenal graft site of new animals.

A panel of thirteen primary adenocarcinoma PCa PDX models (LTL-310F, LTL-311, LTL-313A, LTL-313B, LTL-313C, LTL-313D, LTL-313H, LTL-331, LTL-412, LTL-418, LTL-467, LTL-471, and LTL-484), one CRPC PDX model (LTL-313B), and five NEPC PDX models (LTL-331-8mo, LTL-331R, LTL-352, LTL-370, and LTL-545) were used. In particular, the LTL-313BR CRPC PDX model is derived from the parental LTl-313B adenocarcinoma following recurrence from host castration [220, 228], and the LTL-331R NEPC PDX model is derived from the parental LTL-331 adenocarcinoma following spontaneous NEPC-transdifferentiation after castration [127, 220]. Detailed characteristics of each PDX models, including histological features, proliferation rates, metastatic potentials, and genetic profiles, can be found on the LTL website (http://www.livingtumorlab.com/).

2.2.2 Gene Expression Analysis of PDXs by RNA Microarray

RNA microarray analysis of PDX models were done following protocols previously described [220]. Briefly, 100ng of total RNA was used to generate cyanine-3-labeled cRNA using the one-color Low Input Quick Amp Labeling Kit from Agilent (Santa Clara, CA, USA). The samples were then hybridized on Agilent Sure-Print G3 Human GE 8x60K Microarray Design ID 028004 and scanned with the Agilent DNA Microarray Scanner at 3-µm scan resolution. The data were then processed with Agilent Feature Extraction 11.0.1.1 and quantile normalized with Agilent Gene-Spring 12.0.

2.2.3 Gene Expression Analysis of PDXs by RNA Sequencing

Tumour pieces from LTL-313B and LTL-313BR were also processed for RNA sequencing at the BCCA Michael Smith Genome Sciences Centre according to previously described standard protocols [220]. Briefly, using the Ensembl Release 75 of known gene model annotations, RNA sequencing data reads were first mapped onto the hg19 human reference genome. Exon-exon junctions were mapped by splice-aware aligner STAR [445]. Sequencing reads with unmapped mates or multi-mapped locations were filtered out using Bam Tools [446]. Using the HTSeq tool [447], only reads that were unique to one gene and corresponding exactly with the known gene structure were counted towards the expression of

the corresponding gene. Finally, in order to eliminate the variance arising from differences in sequencing depth among samples, raw read counts were normalized by the DESeq R package [448].

2.2.4 Publically Available PCa Patient Sample Cohorts

Our findings from the PDX models were validated using publically available gene expression datasets of patient PCa samples. For clinical samples of primary treatment-naive PCa, the Memorial Sloan Kettering Cancer Center (MSKCC) cohort consisting of 112 untreated primary PCa adenocarcinomas and 28 samples of benign prostate tissue was used [89]. For clinical samples of CRPC, the University of Michigan cohort consisting of 50 metastatic CRPC samples and 11 high-grade localized PCa samples was used [449]. For clinical samples of NEPC, the Cornell Medical College cohort of six NEPC tumours and 30 PCa adenocarcinomas was used [177].

2.2.5 Metabolic Pathway Scores

A number of prominent cellular metabolic pathways were assessed in this study and included those of glucose, glutamine, lipid, and choline metabolism [450, 451]. Pathways of energy production and macromolecule biosynthesis as previously identified to be significant to PCa cells were also included in our analyses [452-455]. The list of genes comprising each pathway was compiled first by consulting the KEGG Pathways database (http://www.genome.jp/kegg/pathway.html). The gene lists were then refined based on literature reports by, as applicable, dividing larger pathways into smaller component reactions, separating genes predominantly involved in catabolism from those involved primarily in anabolic functions, removing genes of only predicted functionality, and incorporating additional genes with significant experimentally verified contributions that were otherwise not included in the pathways.

Once the genes contributing to each pathway were finalized, a per-gene z-score was calculated based on the mean and standard deviation (SD) of the normalized expression levels of each gene. The per-gene z-scores in each pathway/subpathway were then averaged to arrive at an overall pathway score for each PDX/patient tumour. This method takes into account the contributions of all the genes in a given

pathway, and gives a general indication of up- or down-regulation with an estimated magnitude of difference compared to other tumours. The overall pathway scores of each pathway in the same tumour subtype were then averaged and ranked to give an overall indication of which particular pathways tended to be more or less utilized in a given kind of PCa.

2.2.6 Statistical Analyses

The metabolic pathway scores calculated from the averaged per-gene z-scores provided an overall indication of the direction and magnitude of alteration, suggesting how utilization of each pathway may be changed in a given disease setting. Hierarchical clustering was performed as previously described [456] using the R language (http://cran.r-project.org/). Alterations to glycolysis and lactic acid production pathways were also subjected to t-tests ($p<0.05$, NEPC vs. adenocarcinoma) using GraphPad Prism 6 Software (La Jolla, CA, USA).

2.3 Results

2.3.1 Metabolic Gene Signature in Treatment-naive PCa

Genes from key metabolic pathways were curated from the KEGG pathways database and other literature sources into 23 metabolic processes. While the majority of these pathways are generally involved in bioenergetics and biosynthesis of macromolecules in most cell types, other pathways such as choline and cholesterol metabolism are more specifically relevant to PCa. A schematic representation of these assessed pathways is shown in Figure 2.1, and the full list of genes comprising each pathway can be found in Appendix A. A number of refinements were made in the process of finalizing the gene list in order to better delineate the different processes involved. Notable modifications include the separation of glucose metabolism into the components of glycolysis, gluconeogenesis, pentose phosphate pathway, lactate production, pyruvate conversion to acetyl-CoA, and the TCA cycle; the separation of oxidative phosphorylation into the five complexes of the electron transport chain; and the division catabolic and anabolic processes for choline, ketone bodies, and proline metabolism.

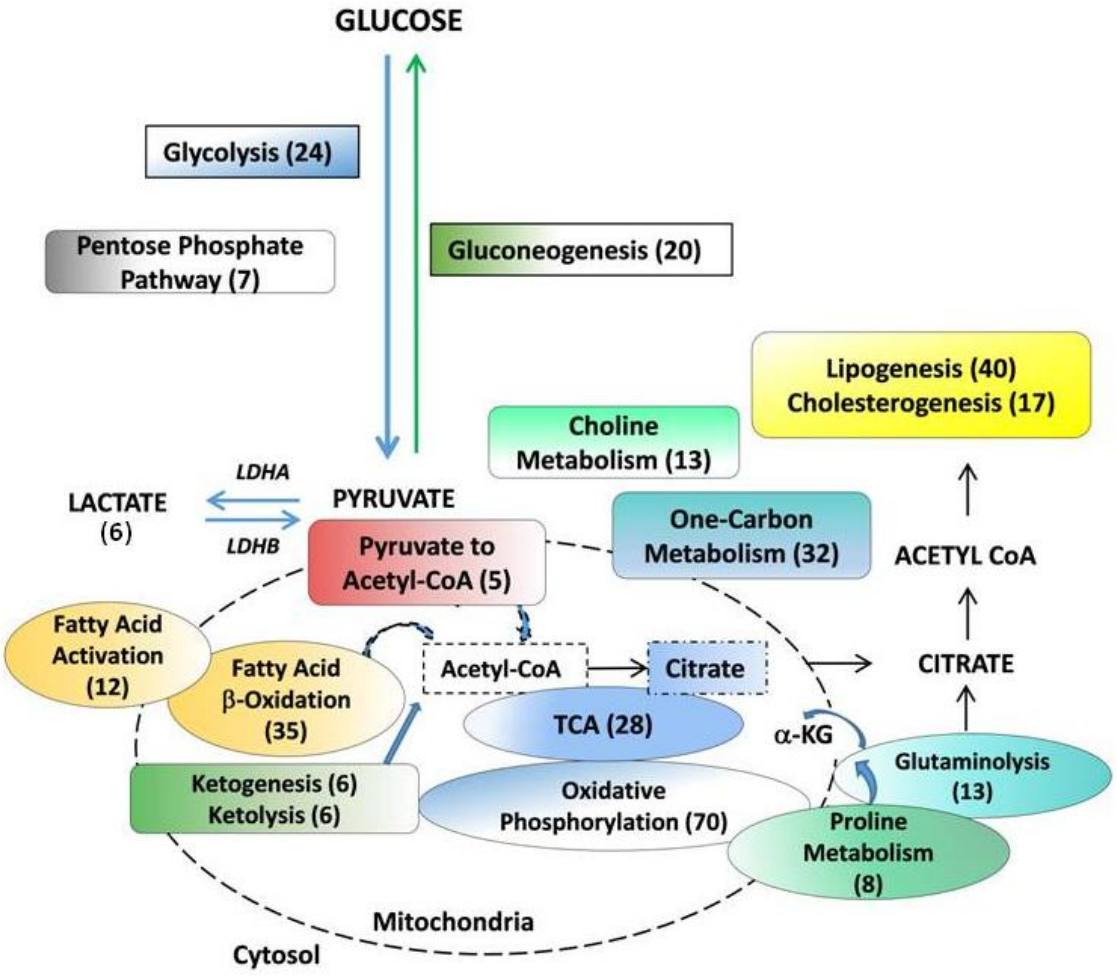

Figure 2.1. A schematic representation of the metabolic pathways assessed in our panel of PCa PDX models and publically available PCa patient datasets. A number of larger metabolic pathways have been divided into small component processes to better delineate the alterations that may be observed and their possible subsequent biological consequences. The majority of these pathways are involved in general bioenergetics or the biosynthesis of macromolecules, while others such as choline and cholesterol metabolism are more specifically pertinent to PCa. The number of genes in each particular pathway is indicated in parenthesis [439].

2.3.1.1 Metabolic Heterogeneity in Treatment-naive PCa PDX and Patients

Gene expression z-scores were calculated using the mean and SD of eleven PDX models derived from primary treatment-naive PCa patients. An analysis of the calculated metabolic pathway scores by hierarchical clustering revealed significant heterogeneity between the PDX models, with mixed up- and down-regulation of various pathways across the different tumour lines (Figure 2.2A). Given the known heterogeneity of primary PCa and the difficulties in disease stratification that such differences present, it is unsurprising that such variations extend to their possible metabolic characteristics as well. For example, the glycolysis and lactic acid production pathways are only upregulated in four (36%) and three (27%) PDX models respectively, suggesting that elevated glycolysis is only relevant to certain primary PCa cases in keeping with literature reports and our hypothesis [436, 437]. This heterogeneity is further evidenced by the clustering pattern of the LTL-313 series of PDXs. While four of the five tumour lines clustered together metabolically, LTL-313A shows a distinct metabolic pattern. As these tumours all originated from different biopsy samples of the same patient prostate, it suggests that metabolic differences can arise even within cancer cells from a single patient's tumour much like the previously reported differences in metastatic potentials [15, 16]. More interestingly, however, is that LTL-331 clustered distinctly from all other tumour lines. As this PDX model is known to spontaneously transdifferentiate into typical NEPC following castration [127], its unique metabolic phenotype compared to other PCa adenocarcinoma PDXs suggests that some of its propensity towards NEPC progression may have metabolic origins.

To verify this metabolic heterogeneity in patient primary treatment-naive PCa tumours, metabolic pathway scores were calculated using the quantile normalized log2 microarray expression data from the MSKCC dataset [89]. Per-gene z-scores for each tumour were generated based on the mean and SD of benign samples. A hierarchical clustering of overall metabolic pathway scores revealed that metabolic heterogeneity is indeed prevalent in patient primary treatment-naive PCa similar to results observed in our PDX models (Figure 2.2B). In particular, dendrogram arms I and II indicate two broadly distinct metabolic phenotypes, with group II exhibiting further heterogeneity as indicated by the subarms i and ii.

Metabolic pathways involved in cholesterol, lipids, and glucose metabolism seem particularly heterogeneous, while pathways of fatty acid activation, ketolysis, and proline metabolism showed less heterogeneity. With regards to our specific interest in glycolysis and lactic acid production, we can confirm that elevated glycolysis is only relevant to a portion of primary PCa patients. Genes in the glycolysis pathway are only upregulated in 26 of 111 (23%) patient samples, whereas genes in the lactic acid production pathway are upregulated in 62 (56%) patient tumours. In contrast, upregulated choline metabolism is much more prevalent, accounting for 93 of 111 (84%) primary PCa cases. This is compatible with clinical observations that $^{18}F/^{11}C$-choline PET is a superior imaging modality compared to FDG-PET in early staging and diagnosis of PCa [457, 458] and lends further confidence that our metabolic pathway scores can accurately reflect tumour metabolic characteristics.

2.3.2 Elevated Glycolytic and Lactate-generating Gene Signature in Advanced PCa

As our analysis on primary treatment-naive PCa PDXs and patients using metabolic pathway scores gave an early indication that this methodology can provide meaningful information regarding tumour metabolic profiles, we proceeded to expand our examination of metabolic gene signatures to include more advanced treatment-resistant subtypes of PCa. There are already some reports in the literature suggesting that an increased reliance on glycolysis could be characteristic of PCa progression into more aggressive subtypes [300, 459]. Furthermore, it has been suggested that androgen nonresponsive PCa cells could exhibit a more glycolytic phenotype compared to their androgen responsive counterparts [460]. Since development of advanced PCa is to a large extent clinically associated with progression from ADT, the identification of altered metabolic pathways following treatment resistance could offer novel insights into potential therapeutic strategies against CRPC and NEPC. The contributions of elevated glycolysis and increased lactic acid production to these tumour types are of particular interest.

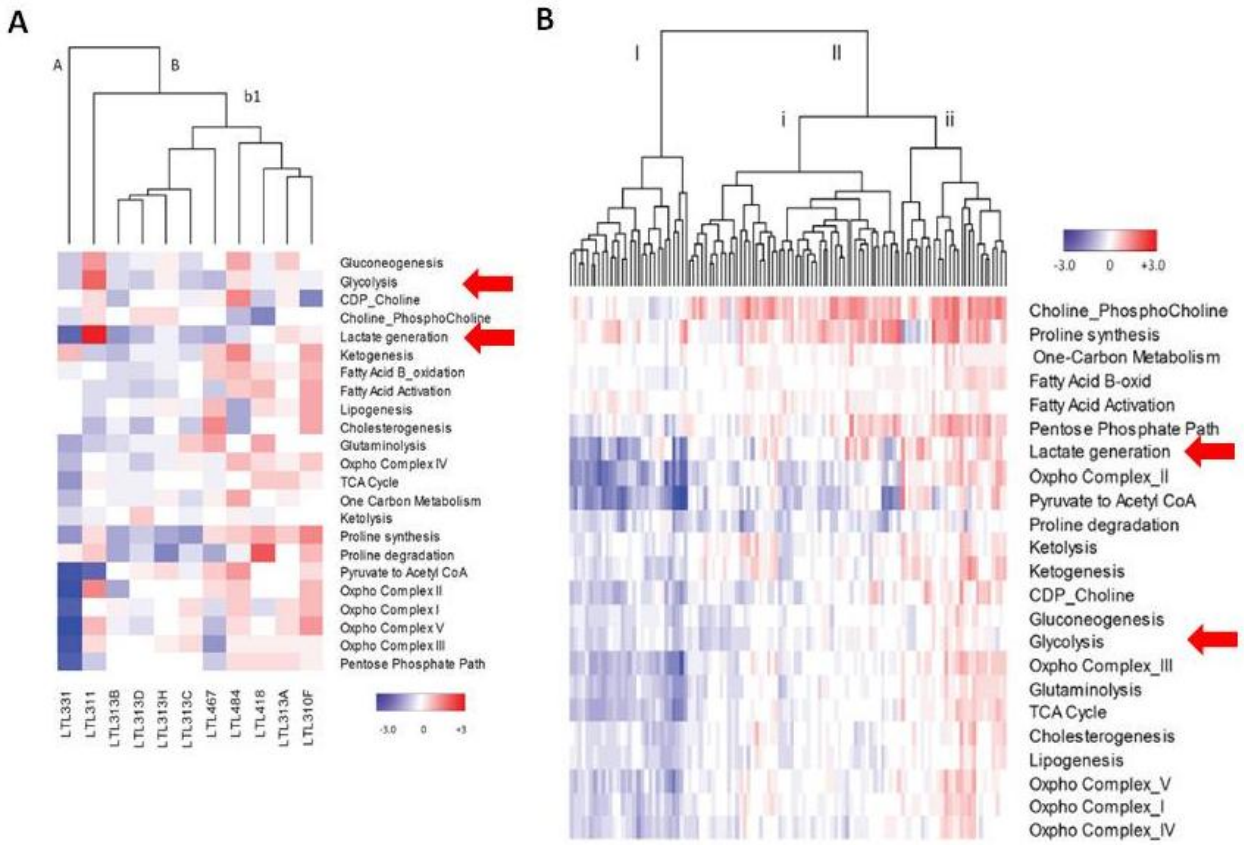

Figure 2.2. Hierarchical clustering analyses of metabolic pathway scores from primary treatment-naive PCa PDXs and patients reveal significant metabolic heterogeneity. For the LTL PDX models (A), z-scores were normalized to all tumours (mean/SD) for each gene and the average z-scores were used to generate pathway scores. For the MSKCC cohort (B), z-scores were normalized to the mean and SD of benign samples for each gene and the average z-scores were used to generate pathway scores. A mix of up- and down-regulated metabolic pathways (coloured red and blue respectively) can be observed in both primary PCa PDX tumour lines (A) and primary PCa patient samples (B), suggesting that significant metabolic heterogeneity is present. In particular, the glycolysis and lactic acid production pathways are only upregulated in certain PDX models and some patient PCa tumours (indicated by the red arrows). In contrast, choline metabolism is more broadly upregulated in patient PCa samples. This is in keeping with clinical observations, both in terms of the heterogeneous nature of the disease and in terms of choline-PET being more useful than FDG-PET for clinical PCa staging and diagnosis. Collectively, this suggests that our metabolic pathway scores can provide accurate estimates of tumour metabolic phenotypes. Dendrogram arms labelled A, B, and b1 (A), and I, II, i, and ii (B) represent various clusters of tumours with potentially distinct metabolic phenotypes. Figure adapted from [439].

2.3.2.1 Elevated Glycolysis and Increased Lactic Acid Production in CRPC PDX and Patients

We have previously developed and characterized a model of CRPC progression using the LTL-313B tumour line. Following host castration, a rapid decrease in serum PSA levels and tumour volume is observed similar to the expected response of clinical PCa tumours. However, the tumour relapses from castration, showing rebounding PSA levels and increasing tumour volumes, ultimately developing into the CRPC tumour line LTL-313BR [220, 228]. A comparison of gene expression between the parental LTL-313B and the castration-resistant LTL-313BR PDX models by RNA-sequencing revealed that a number of genes in the glycolysis and lactic acid production pathways are upregulated following progression to CRPC. In particular, a number of key components of lactic acid production showed greater than 2-fold increased expression, including the lactate transporter MCT4, the primary lactate-producing enzyme LDHA, and the metabolic regulator PDK1, which favours increased lactate generation by inhibiting pyruvate conversion to acetyl-CoA and its subsequent utilization in the TCA cycle. Other proteins involved in glucose metabolism upstream of pyruvate were also found to be upregulated, including various glycolytic enzymes such as PGK1, PGAM1, and ENO1, and the glucose transporter GLUT1 (Figure 2.3A). Taken together, it appears that increased glycolysis and excessive lactic acid production is indeed a metabolic phenomenon associated with castration-resistance.

A similar upregulation of glycolysis and lactic acid production can also be observed in clinical samples of patient CRPC. Using the publically available dataset from the University of Michigan [449], a comparison of gene expression between CRPC and treatment-naive PCa samples showed that, similar to observations from our CRPC PDX models, key proteins and enzymes involved in glycolysis and lactic acid production are more highly expressed in CRPC tumours. More specifically, many of the same key contributors to an enhanced glycolytic and lactic acid-producing phenotype as identified in the LTL-313BR PDX model is also upregulated in CRPC patients, including MCT4, LDHA, PDK1, ENO2, and GLUT1 (Figure 2.3B). As such, not only does this confirm once again that our PDX models can closely mimic patient tumour characteristics in a high-fidelity manner [220], it further suggests that elevated glycolysis and increased lactic acid production is a clinically relevant metabolic feature of CRPC.

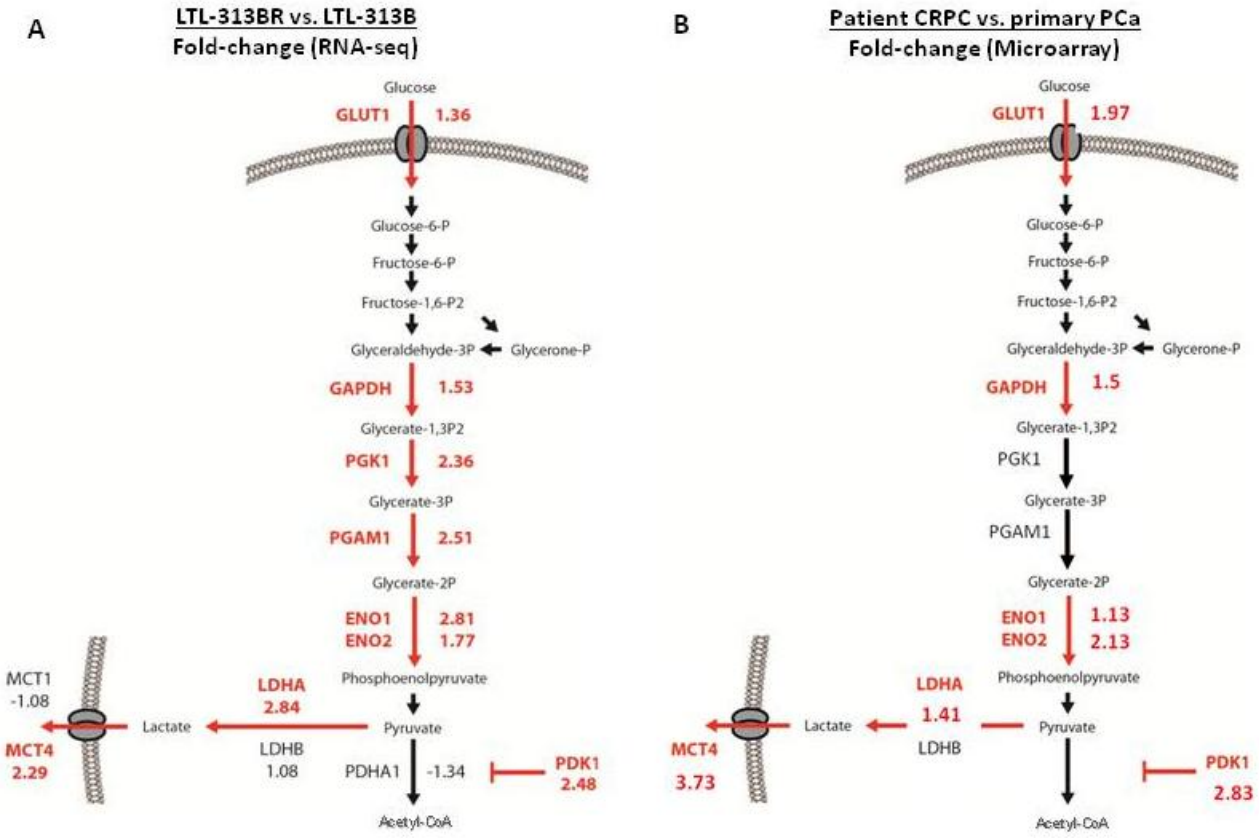

Figure 2.3. Comparison of gene expression between CRPC and primary PCa PDX and patients reveal upregulation of key genes in the glycolysis and lactic acid production pathways. Expression of various genes involved in lactic acid production and upstream glucose metabolism were found to be upregulated in CRPC PDX (A) and patients (B) compared to primary treatment-naive PCa. Various upregulated genes are highlighted in red with the average fold-change between CRPC and primary PCa samples indicated numerically. These genes include the lactate transporter MCT4, the lactate-producing enzyme LDHA, the metabolic regulator PDK1, the glycolytic enzyme ENO2, and the glucose transporter GLUT1. Together, they potentially contribute to elevated glycolysis and increased lactic acid production in CRPC, suggesting that excessive lactic acid generation is indeed a metabolic phenotype clinically relevant to PCa patients as their tumours progress to treatment-resistance.

2.3.2.2 Elevated Glycolysis and Increased Lactic Acid Production in NEPC PDXs and Patients

A more comprehensive assessment of NEPC metabolism was carried out beginning with an initial analysis of the LTL-331 NEPC transdifferentiation model. We have previously demonstrated that the LTL-331 PDX model of PCa adenocarcinoma spontaneously develops into the LTL-331R model of typical $PSA^-/AR^-/SYP^+$ NEPC with small-cell histology following castration [127, 220]. A comparison of metabolic gene expression between the parental LTL-331 PCa adenocarcinoma and transdifferentiated LTL-331R NEPC suggests that elevated glucose metabolism could be potentially relevant to NEPC. By calculating and ranking the various metabolic pathway scores, we found that glucose-related pathways encompassed eight of the nine upregulated metabolic pathways in LTL-331R (Figure 2.4). These NEPC-associated upregulated pathways include glycolysis, lactic acid production, and various complexes involved in oxidative phosphorylation. Interestingly, choline metabolism was most significantly downregulated following NEPC transdifferentiation, suggesting that the elevated choline metabolic phenotype may be a feature specific to PCa adenocarcinoma.

Given the initial indication that glucose metabolism may be relevant to NEPC, we further expanded our analysis to include a panel of five NEPC PDXs and eight PCa adenocarcinoma PDXs. Per-gene z-scores for each NEPC tumour were generated based on the mean and SD of PCa adenocarcinoma PDXs. Metabolic pathway scores from NEPC PDXs were calculated, averaged, and ranked to collectively assess alterations the various pathways. Interestingly, the majority of upregulated metabolic pathways (6 of 8) in this expanded panel of NEPC PDXs remain glucose-related, with glycolysis and lactic acid production ranked second and third, respectively. Choline metabolism was also the most significantly downregulated metabolic pathway in NEPC, confirming initial observations from the LTL-331/331R NEPC transdifferentiation model (Figure 2.5). A hierarchical clustering analysis using the overall metabolic pathway scores was able to distinguish between NEPC and PCa adenocarcinoma PDXs (Figure 2.6A). LTL-331 clustered together with other NEPC PDXs, confirming earlier observations that it is metabolically distinct from other PCa adenocarcinoma PDXs and could, in part, belie its propensity towards NEPC transdifferentiation. More strikingly, a similar hierarchical clustering analysis using the

Metabolic Pathways	331R vs 331Pc Log2 fold change
Proline Degradation	1.154
Glycolysis	0.862
Oxpho Complex I	0.537
Lactate_MCTs	0.534
Pyruvate to Acetyl CoA	0.446
Oxpho Complex V	0.443
Oxpho Complex II	0.426
Gluconeogenesis	0.358
Oxpho Complex III	0.334
One Carbon Metabolism	0.078
Fatty Acid Activation	0.062
Oxpho Complex IV	0.037
TCA Cycle	-0.116
Ketolysis	-0.175
Pentose Phosphate Path	-0.275
CDP-Choline	-0.313
Cholesterogenesis	-0.356
Fatty Acid B oxidation	-0.358
Lipogenesis	-0.440
Glutaminolysis	-0.477
Proline synthesis	-0.508
Ketogenesis	-1.445
Choline-Phosphocholine	-2.770

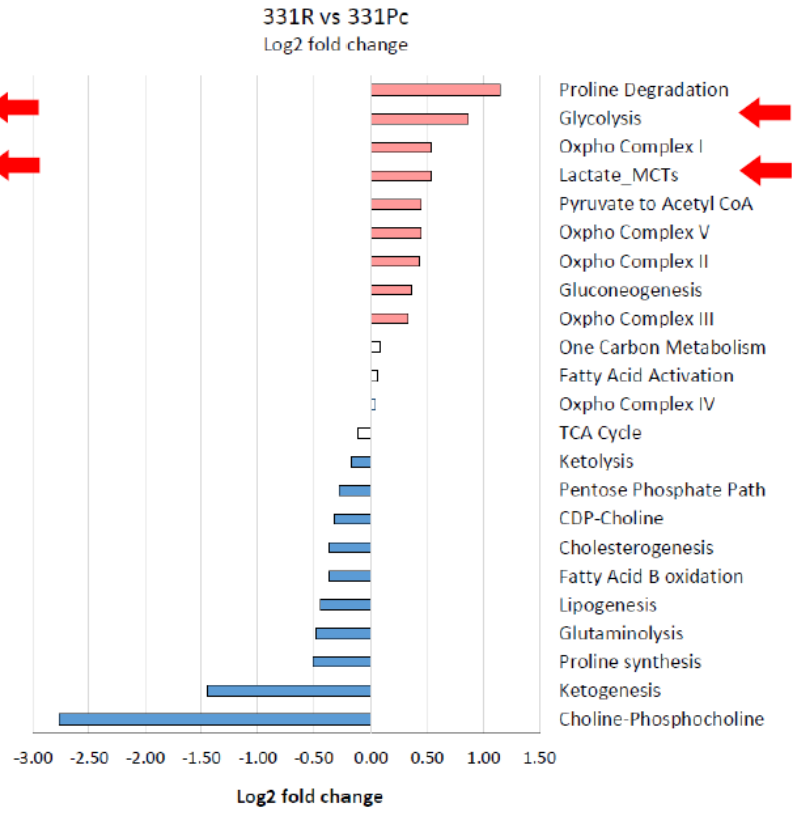

Figure 2.4. A ranking of the metabolic pathway scores comparing the transdifferentiated NEPC PDX model LTL-331R with the parental PCa adenocarcinoma PDX model LTL-331 suggests that elevated glucose metabolism is relevant to NEPC progression. An analysis of the average fold-change of gene expression in various metabolic pathways reveal that glucose-related pathways encompassed eight of the nine upregulated metabolic pathways in LTL-331R, including glycolysis and lactic acid production (indicated by red arrows), and various complexes involved in oxidative phosphorylation. Interestingly, choline metabolism was the most significantly downregulated pathway following NEPC transdifferentiation. The results suggest that an elevated choline metabolic phenotype may be specific to PCa adenocarcinoma. Furthermore, elevated glycolysis and increased lactic acid production could indeed be relevant to NEPC. Upregulated metabolic pathways are indicated in red while downregulated metabolic pathways are indicated in blue.

Metabolic Pathway	Ave NEPC z score
Proline Degradation	0.99
Glycolysis	0.75
Lactate_MCTs	0.61
Gluconeogenesis	0.59
Oxpho Complex IV	0.52
Oxpho Complex I	0.41
Oxpho Complex V	0.39
Fatty Acid Activation	0.35
One Carbon Metabolism	0.03
Oxpho Complex III	-0.11
Cholesterogenesis	-0.18
Lipogenesis	-0.31
Glutaminolysis	-0.33
Oxpho Complex II	-0.38
TCA Cycle	-0.40
Ketolysis	-0.42
Pyruvate to Acetyl CoA	-0.48
CDP-Choline	-0.57
Fatty Acid B oxidation	-0.61
Ketogenesis	-0.79
Pentose Phosphate Path	-1.39
Proline Synthesis	-1.50
Choline-Phosphocholine	-2.28

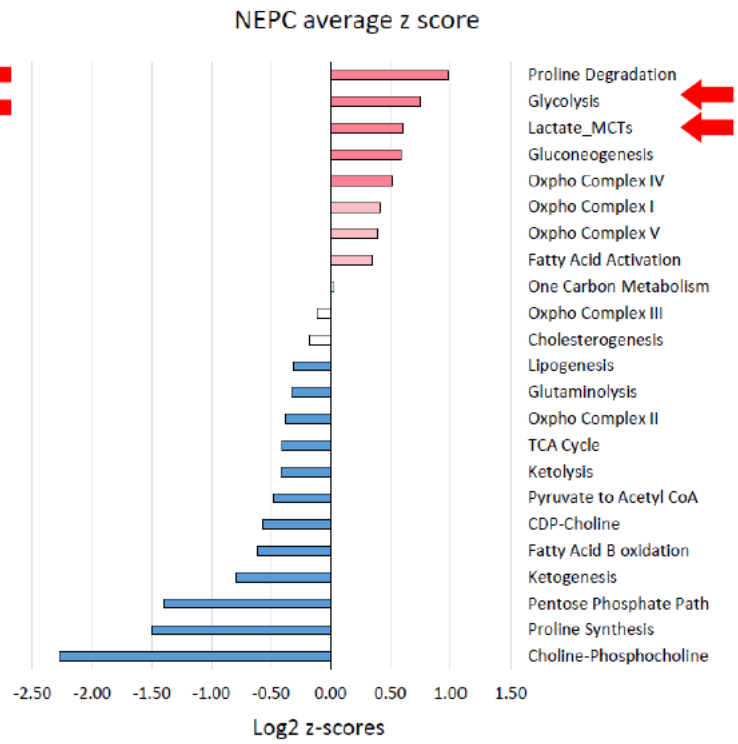

Figure 2.5. A ranking of the metabolic pathways scores comparing an expanded panel of NEPC PDXs (n=5) with PCa adenocarcinoma PDXs (n=8) verifies that glycolysis and lactic acid production pathways are among the top upregulated metabolic pathways in NEPC. Metabolic pathway scores were calculated by averaging the expression z-scores of each gene in NEPC PDXs as normalized to the expression mean and SD of PCa adenocarcinoma PDXs. Once again, glucose-related pathways encompassed the majority (six of eight) of upregulated metabolic pathways in NEPC PDXs, with glycolysis and lactic acid production ranking second and third, respectively (indicated by red arrows). Choline metabolism was also the most significantly downregulated pathway in NEPC PDXs in this expanded analysis. As such, the results confirm earlier observations from the LTL-331/331R NEPC transdifferentiation model and gives further support that elevated glycolysis and increased lactic acid production is important to NEPC. Upregulated metabolic pathways are indicated in red while downregulated metabolic pathways are indicated in blue.

individual genes contributing to the glycolysis and lactic acid production pathways was also able to distinguish between NEPC and PCa adenocarcinoma PDXs (Figure 2.6B). This gives further evidence that elevated glycolysis and increased lactic acid generation could indeed be a distinguishing metabolic phenotype common to NEPC.

Finally, the clinical relevance of our observation that an elevated glycolytic phenotype could be a distinguishing metabolic feature of NEPC was verified using the publically available gene expression dataset of patient NEPC samples from Cornell Medical College [177]. Per-gene z-scores for each pure patient NEPC tumour were generated based on the mean and SD of patient PCa adenocarcinoma, omitting samples containing mixed NEPC and adenocarcinoma histology. Following the same methodology of calculating, averaging, and ranking the overall metabolic pathway scores as previously employed, it is surprising to find that the only upregulated metabolic pathways in NEPC patients are the three pathways with the most direct relationship to glucose metabolism – glycolysis, lactic acid production, and gluconeogenesis (Figure 2.7A). In particular, glycolysis and lactic acid production occupied the top two spots, showing a statistically significantly elevated metabolic pathway score in patient NEPC samples compared to PCa adenocarcinoma (Figure 2.7B). Also of note is that choline metabolism remained the most downregulated metabolic pathway in patient NEPC samples.

A hierarchical clustering using the overall metabolic pathway scores of pure NEPC and PCa adenocarcinoma patient samples was able to distinguish NEPC tumours from PCa adenocarcinoma, validating previous observations from PDX models that NEPC tumours have a distinct metabolic phenotype (Figure 2.8A). Furthermore, using only genes in the lactic acid production pathway, an expanded hierarchical clustering analysis including benign tissue and mixed NEPC/adenocarcinoma was also able to distinguish samples containing NEPC from the other sample types (Figure 2.8B). More intriguingly, while it appears that there is a near-universal upregulation of all genes involved in enhanced lactic acid production, MCT1 (SLC16A1) levels trended in the opposite direction and showed decreased expression in patient NEPC samples. Given that MCT4 (SLC16A3) is much more highly overexpressed, it seems likely that this NEPC-associated metabolic phenotype of elevated glycolysis and increased lactic

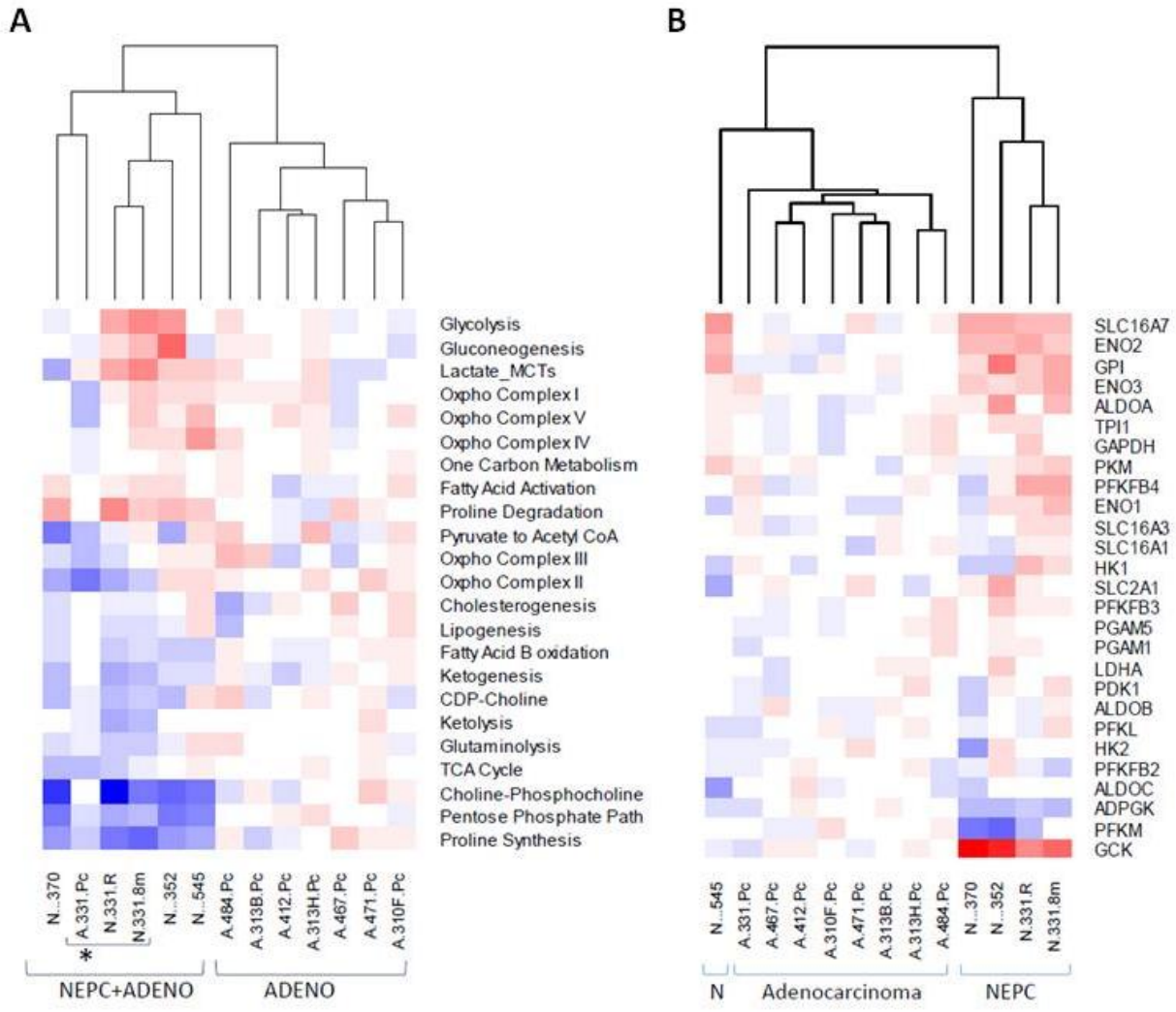

Figure 2.6. Hierarchical clustering analyses of NEPC and PCa adenocarcinoma PDX models further verify the distinct metabolism of NEPC tumours. NEPC PDXs cluster separately from adenocarcinoma PDXs when analyzed with the overall metabolic pathway scores (A) and individual genes specific to the glycolysis and lactic acid production pathways (B). Interestingly, LTL-331 (indicated by *) has a metabolic profile more closely resembling other NEPC PDXs than PCa adenocarcinoma. The analyses give further indication that NEPC tumours have a distinct metabolic phenotype compared to PCa adenocarcinoma, with elevated glycolysis and increased lactic acid generation being one such distinguishing metabolic feature. Upregulated metabolic pathways are indicated in red while downregulated metabolic pathways are indicated in blue.

A

Metabolic Pathways	NEPC Ave z-score
Glycolysis	2.001
Lactate_MCT	1.622
Gluconeogenesis	1.552
One Carbon Metabolism	0.493
Proline Degradation	0.138
Cholesterogenesis	-0.071
Oxpho Complex IV	-0.156
Oxpho Complex I	-0.168
TCA Cycle	-0.187
Ketolysis	-0.238
Lipogenesis	-0.344
Fatty_Acid_Activation	-0.360
Oxpho Complex II	-0.376
Pentose_Phosphate_Path	-0.397
Oxpho Complex III	-0.428
Fatty_Acid_B oxidation	-0.656
Glutaminolysis	-0.733
Oxpho Complex V	-0.776
Pyruvate to Acetyl CoA	-0.846
Ketogenesis	-1.072
CDP_Choline	-1.095
Proline Synthesis	-1.339
Choline_PhosphoCholine	-1.784

B

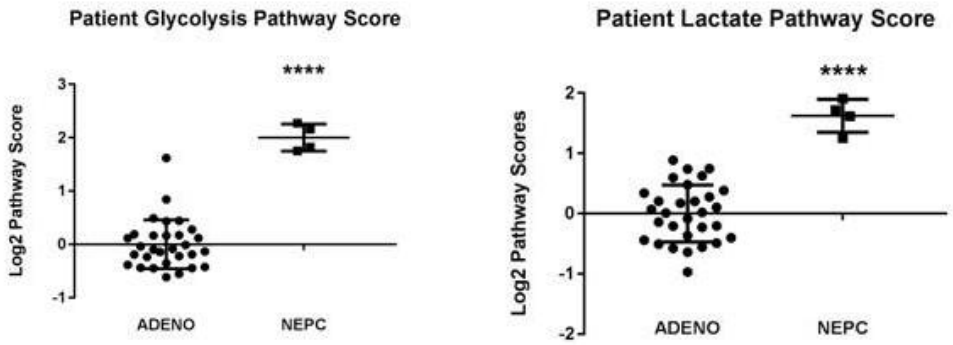

Figure 2.7. An elevated glycolytic gene signature and an associated upregulation of the lactic acid production pathway are even more pronounced in patient NEPC samples. Metabolic pathway scores were calculated by averaging the expression z-scores of each gene in NEPC patient tumours as normalized to the expression mean and SD in PCa adenocarcinoma patient tumours. A ranking of the metabolic pathway scores in patient NEPC tumours reveal that glycolysis and lactic acid production pathways (indicated by red arrows) are the top two upregulated metabolic pathways compared to patient PCa adenocarcinoma samples. Upregulated metabolic pathways are indicated in red while downregulated metabolic pathways are indicated in blue (A). This upregulation is highly statistically significant as assessed by student's t-tests; ****, $p<0.001$ (B). Choline metabolism also remained the most downregulated metabolic pathway in clinical NEPC samples, further validating the previous observations in PDX models as relevant to patients.

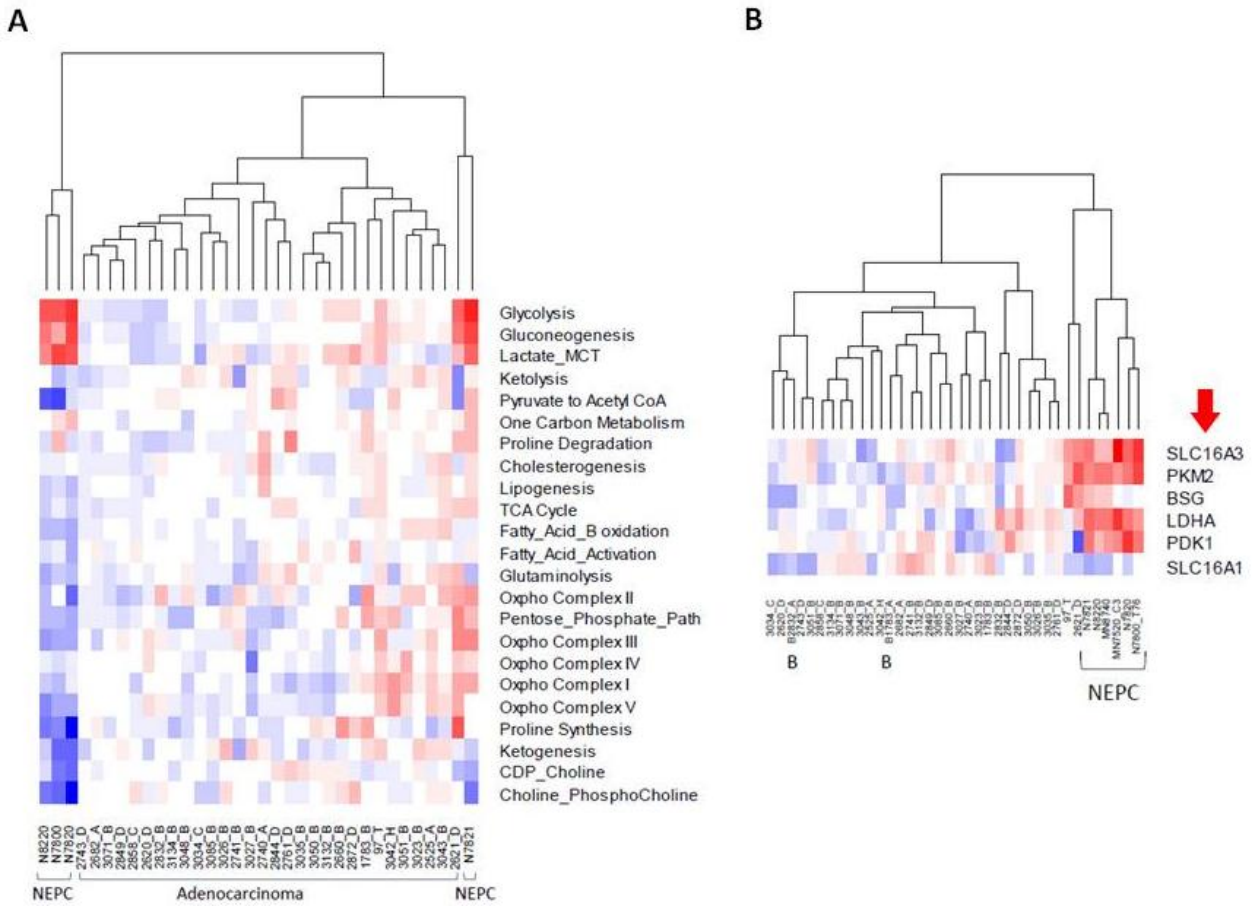

Figure 2.8. The metabolic gene signature observed in patient NEPC tumours is distinct from patient PCa adenocarcinomas samples. A hierarchical clustering analysis using the overall metabolic pathway scores (A) and genes specific to the lactic acid production pathway (B) confirm that patient NEPC tumours have a distinct metabolic phenotype compared to PCa adenocarcinoma. Of particular interest is the significant upregulation of MCT4 (SLC16A3) in NEPC patient samples (indicated by the red arrow). This suggests that it is a clinically relevant mediator of NEPC-associated elevated glycolysis and increased lactic acid production, potentially being a promising therapeutic target. Upregulated metabolic pathways are indicated in red while downregulated metabolic pathways are indicated in blue.

acid production is primarily facilitated through MCT4-mediated lactate export rather than through MCT1 action. As such, targeting MCT4 may be a promising therapeutic strategy to inhibit excessive lactic acid production and secretion in NEPC cells.

2.4 Discussion

By assessing the expression levels of various genes involved in major metabolic pathways of biosynthesis and bioenergetics, we were able to arrive at an estimation of metabolic pathway activity through calculating an overall metabolic pathway score. Utilization of this methodology to assess multiple sample cohorts at various PCa disease stages, including both PDX and patient samples of primary treatment-naive PCa, CRPC, and NEPC, suggests that the metabolic pathway alterations identified by this process is likely to have real implications to tumour biology. Our findings in primary treatment-naive PCa samples coincide with a number of well-established clinical observations. For example, consistent with the known clinical variability already reported from a prognostic [461, 462] and genomic perspective [463, 464], our analysis indicates that primary PCa is also a metabolically heterogeneous disease [439]. Furthermore, localized treatment-naive PCa appear to have a mixed glycolytic profile, with only certain tumours showing upregulated glycolysis and lactic acid production pathways. This is in keeping with clinical observations that FDG-PET is a suboptimal diagnostic tool for assessing and staging early PCa, with only certain tumours showing FDG-PET positivity [440, 465]. Finally, a much larger proportion of patient treatment-naive PCa tumours were found to have upregulated choline metabolism, in line with reports that choline-PET may be a more broadly applicable imaging modality for PCa patients [438, 466]. Taken together, these observations lend confidence that our metabolic pathway scores can accurately reflect PCa tumour metabolism. As such, using this approach to investigate the metabolic phenotype of advanced PCa, of which relatively little is currently known, could potentially uncover meaningful and biologically relevant metabolic characteristics pertinent to CRPC and NEPC.

Assessment of metabolic features in CRPC and NEPC tumours confirmed the close resemblance between PDX models and patient samples, supporting the growing amount of experimental evidence that

the LTL collection of PDX models can accurately mirror patient tumours both in clinical and biological characteristics [127, 200, 220, 467]. We also found initial indications that elevated glycolysis and increased lactic acid production are indeed more relevant to these late-stage, treatment-resistant subtypes of advanced PCa. In cases of CRPC, a number of genes central to facilitating glycolysis and producing lactic acid have increased expression, including MCT4, LDHA, PDK1, ENO2, and GLUT1. This elevated glycolytic and lactate-producing metabolic phenotype is even more prominent in cases of NEPC. Not only can NEPC tumours be distinguished from PCa adenocarcinoma based on the calculated metabolic pathway scores, suggesting a distinct overall metabolic profile, the distinction is evident even when only genes directly involved in glycolysis and lactic acid production are assessed, further indicating that increased aerobic glycolysis contribute significantly to the NEPC metabolic phenotype. More strikingly, elevated glycolysis and increased lactic acid production seem to be the predominant upregulated metabolic pathways in NEPC patient tumours. Collectively, these results confirm our hypothesis in a biologically and clinically relevant manner that PCa tumours indeed become more reliant upon elevated glycolysis and increased lactic acid production as they progress into more aggressive and treatment-resistant subtypes. Furthermore, given the general lack of effective therapies for advanced PCa, inhibiting this increased metabolic dependence upon aerobic glycolysis could be a potentially effective therapeutic strategy for both CRPC and NEPC, especially in view of the multiple tumour-promoting properties of cancer-generated lactic acid.

Despite these promising initial results, a number of limitations inherent to the current methodology should be recognized as areas for improvement in future research endeavours. Firstly, as the primary goal of these analyses was to make better use of existing, previously-generated data to gain novel biological insights, gene expression profiles were used to assess metabolic characteristics. Given that a combination of protein expression, differences in enzyme kinetics, and post-translational modifications such as phosphorylation of key metabolic regulators also contribute to determining ultimate metabolic flux [468, 469], gene expression data is only an indirect indicator of metabolic phenotype. As such, the results here can benefit from confirmation studies using metabolomic approaches for more direct global

assessments or stable isotope tracer experiments to validate more specifically aspects of glucose consumption within tumour cells [470, 471]. Secondly, the research focus of these analyses centred on the involvement of altered glucose metabolism and increased lactic acid production to PCa progression. However, a number of alternate metabolic pathways have also been assessed but are not yet fully appreciated. For example, NEPC tumours seem to show a consistent downregulation of choline metabolism compared to PCa adenocarcinoma samples, indicating that NEPC progression may reverse the broad increase in choline uptake as commonly observed in primary PCa. Additionally, a greater appreciation of the changes to proline metabolism and one-carbon metabolism during PCa progression could yield interesting biological insights and novel therapeutic strategies, particularly given the contributions of proline metabolism to regulating oxidative stress [472] and one-carbon metabolism to epigenetic regulation via DNA methylation [473, 474]. Unfortunately, a more detailed analysis of these pathways lies beyond the scope of this dissertation. Finally, NEPC was chosen as the advanced PCa subtype of focus in these analyses given its status as an emergent, next-generation challenge in clinical PCa management [172, 475]. While the current assessment of CRPC samples is sufficient for the purpose of providing an initial indication of the involvement of elevated glycolysis in advanced PCa, a greater depth of analysis, especially as more data from CRPC PDX models become available, would lend greater confidence to our observations.

In summary, a method to calculate overall metabolic pathway scores was developed to estimate altered utilization of major metabolic pathways during PCa development and progression to treatment-resistance. Analysis of primary treatment-naive PCa samples suggests that primary PCa is a metabolically heterogeneous disease [439] with a mixed glycolytic profile, in keeping with clinical observations regarding inconsistent FDG-PET positivity [440, 465]. Given the mutli-faceted role of cancer-generated lactic acid in promoting various cancer hallmark characteristics, we suspected that an increased reliance on elevated aerobic glycolysis may instead be more relevant to the aggressive, treatment-resistant subtypes of advanced PCa. Analysis of CRPC and NEPC samples confirmed our hypothesis as both biologically and clinically relevant. CRPC PDX and patient tumours showed an increased expression of

genes associated with glycolysis and lactic acid production. Furthermore, upregulated glycolysis and lactic acid production pathways contributed to the distinct metabolic profile of NEPC tumours compared to PCa adenocarcinoma. Interestingly, elevated glycolysis and increased lactic acid production is an even more prominent metabolic phenomenon in NEPC patient tumours. From a therapeutic perspective, an increased expression of MCT4 but not MCT1 can be found in both CRPC and NEPC patient tumours. This suggests that the increased reliance on elevated aerobic glycolysis as observed in advanced PCa tumours is facilitated by MCT4-mediated lactate secretion. As such, inhibition of MCT4 function may be a promising therapeutic strategy for the management of CRPC and NEPC, resulting in the suppression of elevated glycolysis and the reversal of multiple downstream lactate-associated cancer characteristics [343].

Chapter 3: MCT4 Inhibition for Therapy of Advanced PCa – a Proof-of-Concept

3.1 Introduction

Inhibition of MCT4 as a therapeutic strategy has been suggested as potentially promising for the clinical management of a number of different cancer types. In addition to multiple reports of correlations between increased MCT4 expression and poor patient prognosis across a wide range of cancers including breast [369, 476], renal [371], pancreatic [374], lung [477], liver [478, 479], and prostate cancer [382, 480], functional studies have also demonstrated the importance of this lactate transporter in facilitating cancer cell metabolism, proliferation, and other aspects of aggressiveness. Although there is yet no specific SMI targeting lactate export from MCT4, a number of knockdown studies in a variety of cancers have shown promising therapeutic effects. For example, a genome-wide siRNA screen identified MCT4 as important to renal carcinoma cell proliferation and metabolism, with knockdown impairing cell viability and inducing intracellular acidification [481]. A similar siRNA screen in breast cancer cell lines also demonstrated that a reduction in MCT4 expression resulted in a greater reliance on mitochondrial respiration and glutamine metabolism, reversing the Warburg effect [369]. Furthermore, silencing MCT4 expression in oral, lung, and liver cancer cells decreased cell invasion/migration potentials [319, 478, 482], while similar treatments in glioblastoma and lung cancer cells also inhibited cellular response to hypoxia [414, 483, 484]. Taken together, MCT4 appears to play a functionally important role in cancer cells, both in terms of facilitating proliferation via altered cancer metabolism and in terms of promoting additional downstream cancer characteristics as suggested by our hypothesis [343].

Despite this substantial collection of evidence in the literature, the functional role of MCT4 in supporting proliferation and other lactate-associated characteristics in advanced PCa remain relatively unconfirmed. More significantly, the role of MCT4-mediated lactate secretion in creating an immunosuppressive tumour microenvironment has never been studied. Given our earlier assessment using transcriptomic data that elevated glycolysis and increased lactic acid production could be relevant to more advanced, treatment-resistant subtypes of PCa, the biological significance of our findings and the functional relevance of MCT4 in facilitating this metabolic phenotype and its associated downstream

processes need to be experimentally verified. Furthermore, as our previous analysis focused more heavily towards NEPC, a confirmation of the clinical relevance of MCT4 overexpression in CRPC would increase our confidence in its role as a potential therapeutic target. While siRNA is highly effective at decreasing gene expression *in vitro*, its applicability *in vivo* and in clinical settings remain limited. As such, we proceeded to design and validate a panel of human MCT4-specific ASO sequences to identify those effective at decreasing MCT4 expression. Effective candidate MCT4 ASOs were then assessed *in vitro* and *in vivo* in a proof-of-concept study to investigate the potential therapeutic efficacy of MCT4 inhibition for treatment of advanced PCa. The functional relevance of MCT4 in promoting various downstream lactate-mediated oncogenic processes was also assessed, with special emphasis placed upon determining its role in suppressing the anticancer immune response.

3.2 Materials and Methods

All materials and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated.

3.2.1 Construction and Immunohistochemistry (IHC) of Human PCa Tissue Microarray (TMA)

As previously described [16, 485], PCa tissue microarrays (TMA) were manually constructed using various Gleason grades-exhibiting PCa specimens (n = 342) obtained from the Vancouver Prostate Centre (VPC) Tissue Bank with institutional study approval and patients' written informed consent. CRPC samples were obtained via transurethral resection of the prostate, while all other specimens were obtained through radical prostatectomy. IHC staining was conducted using the Discovery XT autostainer (Ventana Medical Systems, Tucson, AZ, USA) paired with an enzyme-labeled streptavidin-biotin system and a solvent-resistant DAB Map Detection Kit (Ventana Medical Systems). Staining intensity on the PCa cell membrane was scored by a trained pathologist on a four-point scale and matched to patient clinical information. A score of 0 represents no staining on any tumor cells; 1 represents a faint or focal,

questionably present staining; 2 represents a convincingly intense stain in a minority of cancer cells; and 3 represents a convincingly intense stain in a majority of cancer cells.

3.2.2 Antibodies

The following antibodies and conjugates were used for western blotting (WB) and IHC: rabbit anti-MCT4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; WB 1:4000, IHC 1:100), mouse anti-vinculin antibody (Sigma; WB 1:1000), rabbit anti-cleaved caspase 3 antibody (Cell Signalling Technology, Danvers, MA, USA; IHC 1:50), mouse anti-Ki67 antibody (Dako, Burlington, ON, Canada; IHC 1:50), rat anti-CD31 antibody (Dianova, Hamburg, Germany; IHC 1:20), mouse anti-pan-T cell marker CD3 antibody (Dako; IHC 1:50), biotinylated mouse anti-NK1.1 (Cedarlane, Burlington, ON, Canada; IHC 1:100), mouse anti-human CD45 antibody (Dako; IHC 1:400), rabbit anti-human CD8 antibody (Abcam, Cambridge, MA, USA; IHC 1:500), IRDye 800CW goat anti-mouse antibody (LI-COR Biosciences, Lincoln, NE, USA; WB 1:10,000), IRDye 680RD goat anti-rabbit antibody (Li-Cor Biosciences; WB 1:10,000), biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA; IHC 1:200), biotinylated goat anti-rat antibody (Vector Laboratories; IHC 1:200), and biotinylated goat anti-mouse antibody (Vector Laboratories; IHC 1:200).

3.2.3 MCT4 ASO Design and Selection

Potential ASOs against human MCT4 were rationally designed by identifying favourable motifs along the mRNA transcript while avoiding unfavourable sequences [486]. ASO specificity to human MCT4 was defined as at least 3 of 20 mismatched bases to other human and mouse genes when assessed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For experimental purposes, ten sequences distributed throughout the length of the human MCT4 transcript with perfect complementarity to all transcript variants (NM_001042422.2, NM_001042423.2, NM_001206950.1, NM_001206951.1, NM_001206952.1, and NM_004207.3) were selected and synthesized with first-generation PS-backbone modifications by Eurofins MWG Operon (Huntsville, AL, USA). Two candidate MCT4 ASOs (#1 and

#14) were selected for further studies. The sequences are as follows: ASO #1, 5'-TCCCATGGCCAGGAGGGTTG-3'; ASO #14, 5'-AGATGCAGAAGACCACGAGG-3'; a published non-targeting control ASO, 5'-CCTTCCCTGAAGGTTCCTCC-3' [487, 488]. For patent purposes, an expanded panel of 50 potential MCT4 ASOs were assessed. This larger panel consists of new sequences, the originally tested MCT4 ASOs, and shortened 16-nucleotide derivatives of ASO#1 and ASO#14. See Appendix B for a full listing of tested sequences.

3.2.4 PCa Cell Cultures

Human PC-3 and DU145 CRPC cells, human LNCaP PCa cells, human NCI-H660 NEPC cells, and mouse TRAMP-C2 PCa cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). C4-2 CRPC cells were obtained from Dr. Martin E. Gleave (Vancouver Prostate Centre, Vancouver, BC, Canada). Human monolayer cell cultures were maintained in RPMI-1640 (GE Healthcare HyClone, Logan, UT, USA) supplemented with 10% FBS (GE Healthcare HyClone), whereas TRAMP-C2 cells were maintained in DMEM (GE Healthcare HyClone) supplemented with 5% FBS. NCI-H660 NEPC cells were maintained in RPMI-1640 following the protocol provided by ATCC. For cell counting, cultures were trypsinized to form a single-cell suspension and counted using a Bio-Rad TC20 Automated Cell Counter (Hercules, CA, USA). Cell viability was assessed by trypan blue exclusion.

3.2.5 ASO and siRNA Transfection

ASO transfections were carried out at 100nM for 48 hours unless otherwise indicated using Oligofectamine (Invitrogen, Carlsbad, CA, USA) on cells seeded in 6-well plates. MCT4-targeting siRNA and controls (Dharmacon, Chicago, IL, USA) were transfected at 50nM for 48 hours using Lipofectamine 2000 (Invitrogen) under similar conditions. NCI-H660 cells were transfected in serum-free media using Lipofectamine RNAiMAX (Invitrogen). All transfections were carried out according to the manufacturer's instructions.

3.2.6 Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was isolated from harvested cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified using the NanoDrop 2000c (ThermoFisher Scientific, Waltham, MA, USA). A total of 1µg of RNA was used for cDNA synthesis with the QuantiTect Reverse Transcription Kit (Qiagen). Primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Samples were loaded on a 384-well plate in triplicates with SYBR FAST Universal qPCR Master Mix (KAPA Biosystems, Woburn, MA, USA) and quantified using the ViiA-7 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Target genes were normalized to a geometric average of three reference genes [489]. See Appendix C for a full listing of qPCR primer sequences.

3.2.7 Western Blotting

Cell samples were harvested and lysed in RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with a complete protease inhibitor cocktail (Roche, Nutley, NJ, USA). Protein concentrations were determined by the BCA Protein Assay (ThermoFisher Scientific). 20 µg of lysate was run on an 8% SDS-polyacrylamide gel and transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The blot was blocked with the Odyssey Blocking Buffer (LI-COR Biosciences) and probed with anti-MCT4 antibody. Vinculin was also probed as a loading control. The primary antibodies were incubated at 4°C overnight and the corresponding secondary antibody was added the following day. The Odyssey Infrared Imaging System (LI-COR Biosciences) and Image Studio Version 3.1 (LI-COR Biosciences) was used for detection.

3.2.8 Modified Boyden Chamber Assay

The migrating and invasive abilities of PC-3 cells following MCT4 ASO treatments were assessed using Matrigel-coated modified Boyden chambers (BD Biosciences, San Jose, CA, USA) as previously described [16]. Briefly, ASO-treated PC-3 cells were seeded at 50,000 live cells per well into the top chamber in serum-free media. Media with FBS as the chemoattractant was added to the lower

chamber. After 48 hours, cells that have migrated/invaded into the lower chamber were dissociated and resuspended using dissociation buffer (Trevigen, Gaithersburg, MD, USA) containing 12.5 mM calcein-AM (Trevigen). Triplicate aliquots of the cell suspension were measured by fluorescence (485nm excitation; 520nm emission) using the Infinite F500 fluorometer (Tecan, Männedorf, Switzerland) to determine the total number of migrated/invaded cells.

3.2.9 Determination of Lactate Secretion and Glucose Consumption

Changes to glucose metabolism following MCT4 ASO treatment were characterized by assessing lactic acid secretion and glucose consumption in ASO-treated cells 48 hours post-transfection. Cells were incubated in fresh media for 4 hours before media samples were taken. The samples were then deproteinated using 10K Spin Columns (BioVision, Milpitas, CA, USA). Lactate concentrations were measured using the Lactate Assay Kit (BioVision) and glucose concentrations were measured using the Glucose Assay Kit (BioVision). Intracellular lactate levels were determined by lysing ASO-transfected cells in MQH_2O via repeated freeze-thaw cycles. Overall glucose consumption and lactic acid secretion were normalized to the total number of live cells.

3.2.10 Treatment of PC-3 Tumour-bearing Nude Mice with MCT4 ASOs

One million PC-3 cells in 1:1 HBSS:Matrigel were injected subcutaneously into both flanks of 24 male athymic nude mice (Simonsen Laboratories, Gilroy, CA, USA). The mice were randomized into four groups once mean tumour volume reached 100 mm^3 and were treated with intraperitoneal injections of vehicle (PBS), control ASO, MCT4 ASO #1, or MCT4 ASO #14 at 10 mg/kg daily for 5 days followed by 2 days off treatment for a total duration of 15 days. Health monitoring was done throughout the study by checking for abnormal behaviors (such as lack of hydration, lethargy, and further signs of weakness) and measuring body weights. Tumour size was measured twice weekly for the calculation of tumour volume using the formula: volume (mm^3) = length (mm) × width (mm) × depth (mm) × 0.5236. Mice were sacrificed for tissue harvesting one hour after the final dose.

3.2.11 Treatment of First-generation PDX with MCT4 ASOs

Fresh patient PCa tissue from a lymph node metastasis was obtained from the Vancouver General Hospital following patient informed consent. The tumour was divided into smaller tissue pieces and grafted under the renal capsule of NOD-SCID mice supplemented with testosterone as previously described [218, 220]. Four tumour pieces were grafted per animal with two pieces per kidney. Ten days after grafting, the mice were treated with intraperitoneal injections of control ASO or MCT4 ASO #14 at 15mg/kg daily for 5 days followed by 2 days off treatment for a total duration of three weeks. The mice were euthanized at the end of the treatment schedule for tissue harvesting.

3.2.12 Assessment of Tumour Tissues by IHC

IHC analyses were done on formalin-fixed, paraffin-embedded tumour tissues. Tissue blocks were sectioned, probed by various antibodies of interest, and stained with DAB as previously described [490]. Changes to tumour cell proliferation and apoptosis after treatment were assessed by Ki-67 and cleaved-caspase 3staining respectively. Five random fields at 400× magnification were imaged per tumour and total cancer cells were counted to determine the percentage of positively stained cells. For assessment of MCT4 knockdown *in vivo*, images of five random fields at 200× magnification were taken per tumour and assessed by percentage scoring for average staining intensity using the formula: Intensity = (% area score 3) × 3 + (% area score 2) × 2 + (% area score 1) × 1. The extent of immune cell extravasation and aggregation was quantified following CD31 staining of endothelial cells. The five most prominent regions per tumour containing tightly-packed, small, round nuclei in the vicinity of CD31-positive blood vessels were imaged at 200× magnification to determine the percent area of the field they occupied. The proportions of immune cell subtypes were evaluated using images of the same five prominent regions from serial sections and were calculated as the area of positive marker staining (NK1.1 or CD3) normalized to the area occupied by immune cell aggregates.

For first-generation PDXs, residual tumour percentage was calculated as the proportion of remaining live tumour tissue within the overall graft area. Proliferative patient tumour-associated immune

cells were defined as human Ki67-positive cells showing a small, round, densely-packed morphology. The percentage of these proliferative immune cells compared to the total immune cells present was estimated by a trained pathologist. The presence of specific immune cell subtypes was evaluated by counting the total number of human marker-positive cells (CD45 or CD8) in ten randomly selected high-power fields (400× magnification) per tumour.

3.2.13 Statistical Analysis

Statistical analysis was carried out using the GraphPad Prism 6 software. Student t-tests were used to compare means between two groups while one-way ANOVA followed by post-hoc Dunnett's tests were carried out to compare means of more than two groups. Tumour growth *in vivo* was analyzed by two-way ANOVA with post-hoc multiple comparison. A contingency test was used to compare staining intensity on the TMA among patient cohorts, and patient survival curves were compared with a log rank test. All averaged results are represented graphically as mean ± SEM. Results with $p < 0.05$ were considered statistically significant and are represented by * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and **** for $p < 0.0001$.

3.3 Results

3.3.1 Elevated MCT4 Protein Expression Observed in CRPC Patient Tumours

It has already been reported in the literature that elevated MCT4 expression is correlated with a number of clinicopathological parameters associated with poor PCa patient prognosis, including higher PSA levels, Gleason grades, and clinical pT stages [381]. Furthermore, increased incidences of perineural invasion and biochemical recurrence have also been reported for PCa patients with high MCT4-expressing tumours [480]. MCT4 staining in the VPC collection of patient PCa tumour samples confirmed such literature reports, revealing that high membrane MCT4 expression is indeed associated with poor prognosis. In particular, significantly higher MCT4 expression was found in Gleason grade 5 specimens compared to tumours of Gleason grades 3 and 4. This was true both in terms of average

staining intensity and proportion of high MCT4-expressing tumours (intensity scores 2 and 3) (Figure 3.1A&B). Additionally, elevated MCT4 expression is associated with an earlier time to relapse in this patient cohort, as measured by increases in serum PSA following primary treatment. Patients with high MCT4-expressing tumours (intensity scores 2 and 3) had a median time to relapse of 63.3 months while patients with low MCT4-expressing tumours (intensity scores 0 and 1) had a median time to relapse of 94.2 months (Figure 3.1C). More interestingly, increased MCT4 protein expression was also found in tumour samples from patients subjected to prolonged (>6 months) neoadjuvant hormone therapy (NHT) and patients with CRPC (Figure 3.1D&E). This elevated MCT4 expression in CRPC tumours represents a novel and previously unreported observation, confirming our earlier transcriptomic analysis indicating that increased MCT4 could facilitate elevated glycolysis and increased lactic acid secretion during CRPC progression. As such, the effective inhibition of MCT4 could prove to be a clinically relevant therapeutic strategy for the treatment of advanced PCa, including both CRPC and NEPC.

3.3.2 Efficacy of MCT4 Inhibition *in vitro*

Given that MCT4 is confirmed as a clinically relevant therapeutic target for both CRPC and NEPC patients, we proceeded to assess the efficacy of MCT4 inhibition as a treatment strategy. As there are currently no isoform-specific SMIs inhibiting lactate export from MCT4, we designed therapeutic MCT4-targeting ASOs for use in both *in vitro* and *in vivo* settings. In addition to assessing changes to cell proliferation following successful MCT4 knockdown, we also investigated the effects of decreased MCT4 expression to various downstream lactate-associated oncogenic processes to generate initial proof-of-concept evidence in support of our hypothesis and proposed therapeutic strategy.

3.3.2.1 MCT4 ASOs Specifically and Effectively Decrease Human MCT4 Expression

A panel of ten potential MCT4-targeting ASOs with sequence complementarity dispersed throughout the length of the MCT4 transcript were designed and synthesized for an initial assessment of efficacy. Changes to MCT4 and MCT1 expression were assessed 48 hours after transfecting 100nM of

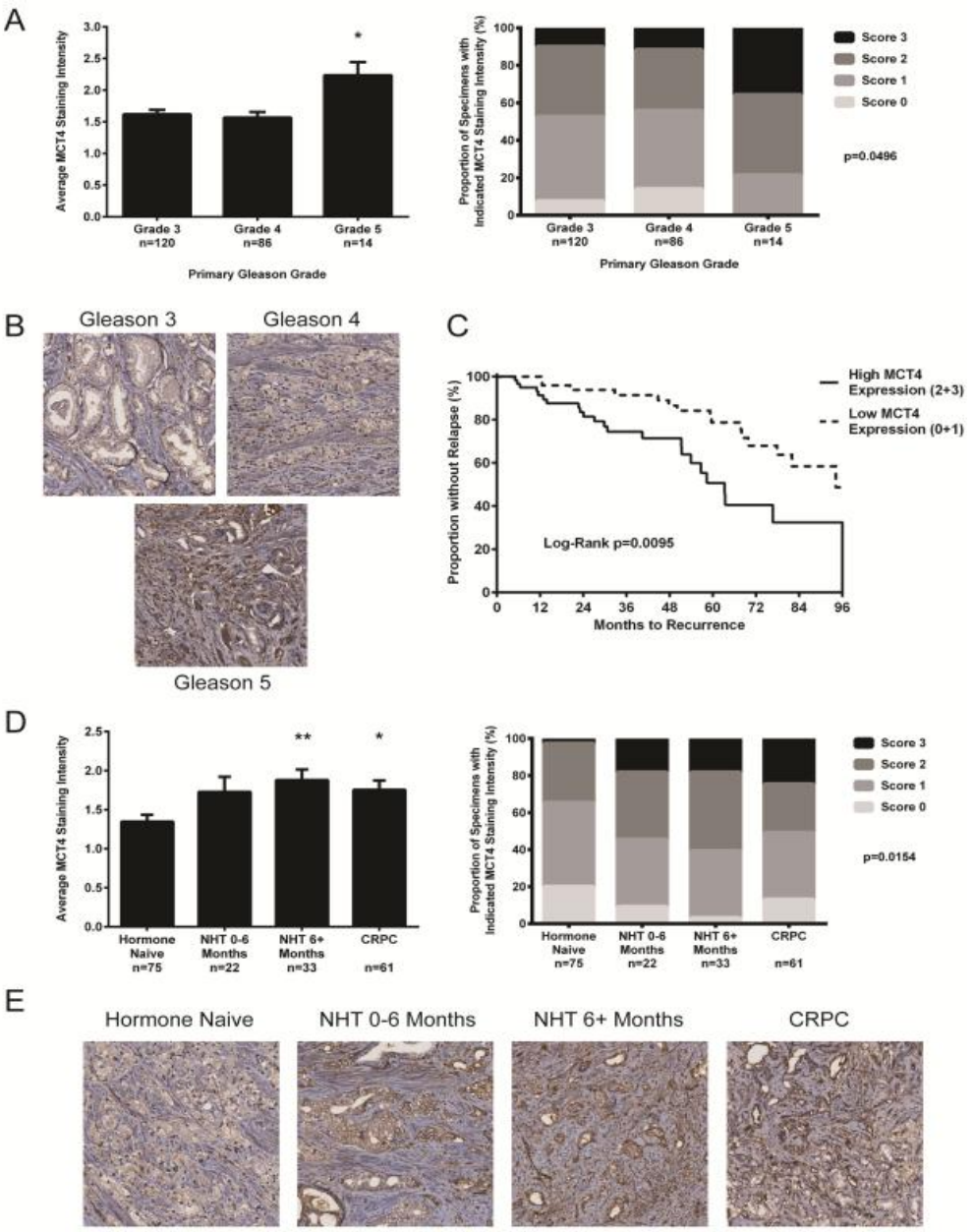

Figure 3.1. Elevated MCT4 expression is clinically relevant to CRPC patients and represents a novel therapeutic target. TMAs constructed from PCa tumour samples available at the VPC were stained for MCT4 and scored on a four-point scale for membrane staining intensity by a trained pathologist. The average staining intensities reveal that elevated MCT4 expression is associated with higher Gleason grade (A), as observed in representative images showing greater staining intensity in Gleason grade 5 specimens (B). High MCT4 expression is also associated with an earlier time to relapse (C). Patients undergoing prolonged NHT and those with CRPC also show increased MCT4 expression (D), as evident in representative images of the corresponding tumours (E). Statistical analyses were done using one-way ANOVA with post-hoc Dunnett's test; *, $p < 0.05$; **, $p < 0.01$. Figure reproduced from [382].

ASOs into PC-3 cells. As expected, the ASOs were able to decrease MCT4 mRNA and protein expression at varying efficacies, with MCT4 ASO #1 and #14 being the most effective (Figure 3.2A). Importantly, the potential MCT4 ASOs did not significantly affect MCT1 expression (Figure 3.2B), providing an initial indication that these ASOs are MCT4-specific. Treatment of PC-3 cells with MCT4 ASOs also inhibited cell proliferation (Figure 3.2C) similar to inhibition resulting from MCT4 siRNA treatment (Figure 3.2D). Significantly, there is a strong correlation between the inhibition of cell proliferation and the extent of MCT4 knockdown as achieved by the various ASO sequences, with greater MCT4 knockdown resulting in more drastic reductions in cell proliferation (Figure 3.2E). Additionally, transfection of the effective MCT4 ASOs #1 and #14 into mouse TRAMP-C2 PCa cells decreased neither mouse cell proliferation nor mouse MCT4 expression (Figure 3.2F). Taken together, these results suggest that the MCT4 ASOs are indeed specific against human MCT4.

Given the initial results suggesting that MCT4 ASOs can be effective at both decreasing MCT4 expression and inhibiting PCa cell proliferation, an expanded panel of ASOs were designed and tested for patent application purposes. The full panel was reassessed at an increased dose of 250nM to cover all potentially effective sequences. Of the 50 tested sequences, 23 were able to decrease MCT4 expression by greater than 50% without affecting MCT1 expression. The shortened 16-nucleotide variants of MCT4 ASO#1were also able to effectively reduce MCT4 expression, whereas a previously published sequence targeting rat MCT4 [491] was not able to reduce human MCT4 expression (Table 3.1). The sequences were subsequently included in a patent application for future commercialization and potential clinical use.

3.3.2.2 MCT4 Knockdown Reduces Advanced PCa Cell Proliferation

The effective MCT4 ASOs #1 and #14 were further characterized. A dose-dependence analysis in PC-3 cells revealed that both ASOs have similar efficacies, with the IC_{50} of ASO#1 being 50nM and the IC_{50} of ASO#14 being 26nM. Importantly, the inhibition of cell proliferation closely mirrored decreased MCT4 expression, showing comparable IC_{50} values (Figure 3.3A). This suggests that MCT4 expression is biologically important in facilitating proliferation of advanced PCa cells. Both candidate ASOs were able

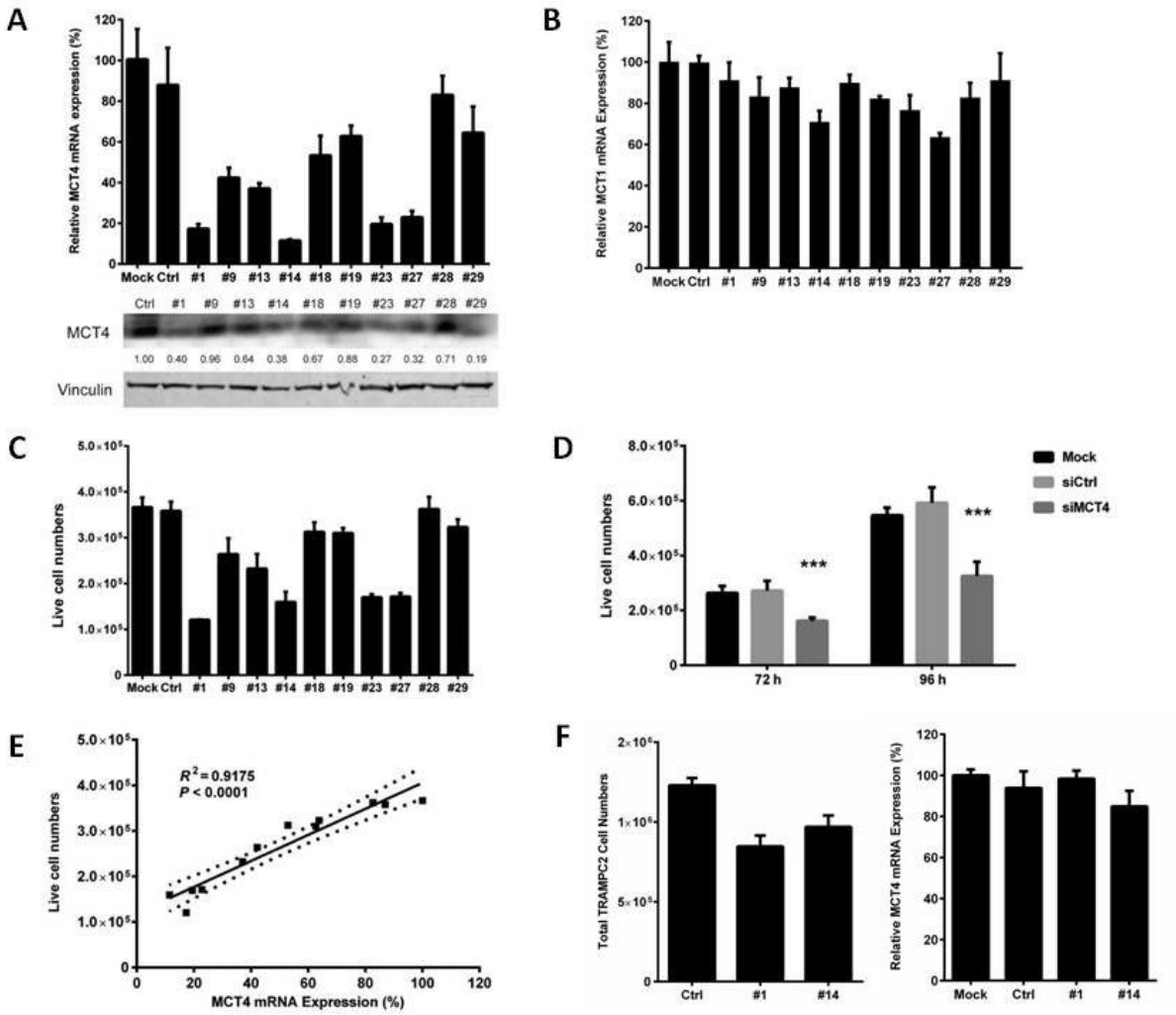

Figure 3.2. MCT4-targeting ASOs are effective and specific at decreasing human MCT4 expression. Transfection of ten MCT4 ASOs into PC-3 cells reveals varied efficacy at decreasing MCT4 mRNA and protein expression 48 hours after transfection (A) without affecting MCT1 levels (B), with MCT4 ASO#1 and #14 being the most effective. Furthermore, MCT4 ASOs were also able to decrease PC-3 cell proliferation to various extents (C) similar to inhibitions of cell proliferation observed following MCT4 siRNA treatment. Statistical significance was assessed by one-way ANOVA with post-hoc Dunnett's test; ***, $p < 0.001$ (D). More importantly, a linear regression analysis showed a strong correlation ($p < 0.0001$) between the MCT4 knockdown induced by the different ASO sequences and the resultant inhibition in cell proliferation (E). Furthermore, mouse TRAMP-C2 PCa cell proliferation and mouse MCT4 mRNA expression remain unaffected by the most effect MCT4 ASO sequences (F). Taken together, the MCT4 ASOs are effective at decreasing MCT4 expression and specific against human MCT4. Figure reproduced from [382].

Table 3.1. Summary of the expanded panel of 50 MCT4 ASO sequences tested for patent application purposes. An increased dose of 250nM was transfected into PC-3 cells and a greater than 50% reduction in MCT4 expression 48 hours post-transfection was considered effective (patented MCT4 ASOs are highlighted). All values were normalized to levels after scrambled non-targeting control ASO treatment.

MCT4 ASO	MCT4 Knockdown (%)	MCT1 Knockdown (%)	MCT4 ASO	MCT4 Knockdown (%)	MCT1 Knockdown (%)
Rat	22.40	16.02	**#20**	**78.67**	-7.98
#1	**65.52**	-1.67	**#21**	**66.31**	-1.95
#1v1 (16 nt)	**76.03**	-36.99	**#22**	**80.77**	-5.28
#1v2 (16 nt)	**81.21**	-25.48	**#23**	**76.03**	17.08
#1v3 (16 nt)	**77.65**	-57.78	**#24**	**76.51**	-23.60
#1v4 (16 nt)	**65.76**	-33.80	#25	45.92	-72.66
#1v5 (16 nt)	30.52	-43.10	#26	39.46	-30.53
#2	47.24	-2.49	**#27**	**63.01**	12.56
#3	**86.33**	12.91	#28	-42.05	-8.77
#4	22.88	-12.66	#29	17.02	5.64
#5	9.55	1.74	#30	21.20	16.02
#6	3.17	4.88	#31	0.27	9.93
#7	29.91	4.57	**#32**	**70.36**	16.57
#8	16.31	35.47	#33	31.16	-7.61
#9	25.51	-8.40	**#34**	**66.85**	35.73
#10	-43.40	-24.34	**#35**	**72.00**	29.19
#11	18.67	-16.93	**#36**	**75.58**	47.09
#12	**71.19**	-25.86	**#37**	**55.47**	-0.16
#13	**77.13**	**55.61**	**#38**	**70.93**	-22.12
#14	**78.16**	24.93	**#39**	**71.14**	24.45
#15	49.00	-40.40	**#40**	**80.26**	30.66
#16	20.50	-30.53	**#41**	**66.26**	23.17
#17	-10.07	-22.51	**#42**	**60.36**	3.36
#18	9.33	-3.87	**#43**	**79.96**	7.63
#19	33.14	-1.77	**#44**	**65.41**	21.78

to sustain the inhibition of cell proliferation and reduction of MCT4 expression up to 96 hours post-treatment. While a slight increase in MCT4 mRNA levels can be observed beginning at 72 hours after treatment, MCT4 protein levels remained low even at 96 hours post-transfection, thus maintaining the anti-proliferative effects of the ASOs (Figure 3.3B).

Similar inhibitions to cell proliferation as a result of MCT4 knockdown by our candidate ASOs can also be observed in other highly glycolytic, advanced PCa cell lines. Intriguingly, the efficacy of MCT4 ASOs in the other CRPC cell lines C4-2 (Figure 3.4A) and DU145 (Figure 3.4B) resemble that observed in PC-3 cells, showing relatively consistent IC_{50} values both in terms of MCT4 knockdown and inhibition of cell proliferation. A reduction in cell growth following MCT4 knockdown was also observed in the LNCaP cell line (Figure 3.4C). Collectively, these results give further evidence that MCT4 expression plays a functionally important role across multiple types of advanced PCa cells and is closely associated with their proliferative abilities.

3.3.2.3 MCT4 Knockdown Inhibit Invasion/Migration and Block Glucose Metabolism

While sustained proliferation is a key cancer hallmark facilitated by MCT4 action, other tumour-promoting characteristics fundamental to cancer biology can also be significantly affected. According to our hypothesis and reports in the literature [312, 319, 482], cancer-generated lactic acid can induce tissue invasion and distant-organ metastasis. Furthermore, a more detailed investigation into the potential effects of MCT4 inhibition on upstream glucose metabolism can also help better understand the mechanism of action of our candidate MCT4 ASOs.

To investigate changes to migration/invasion potentials and alterations to glucose metabolism following MCT4 knockdown, PC-3 cells were once more transfected with 100nM of MCT4 ASO#1 and #14. Modified Boyden chambers with or without Matrigel coating were used to assess invasion and migration potentials respectively. MCT4 ASO-treated PC-3 cells were found to have significantly inhibited transwell invasion and migration capabilities (Figure 3.5A), suggesting that MCT4 action is indeed important to facilitating the metastatic process.

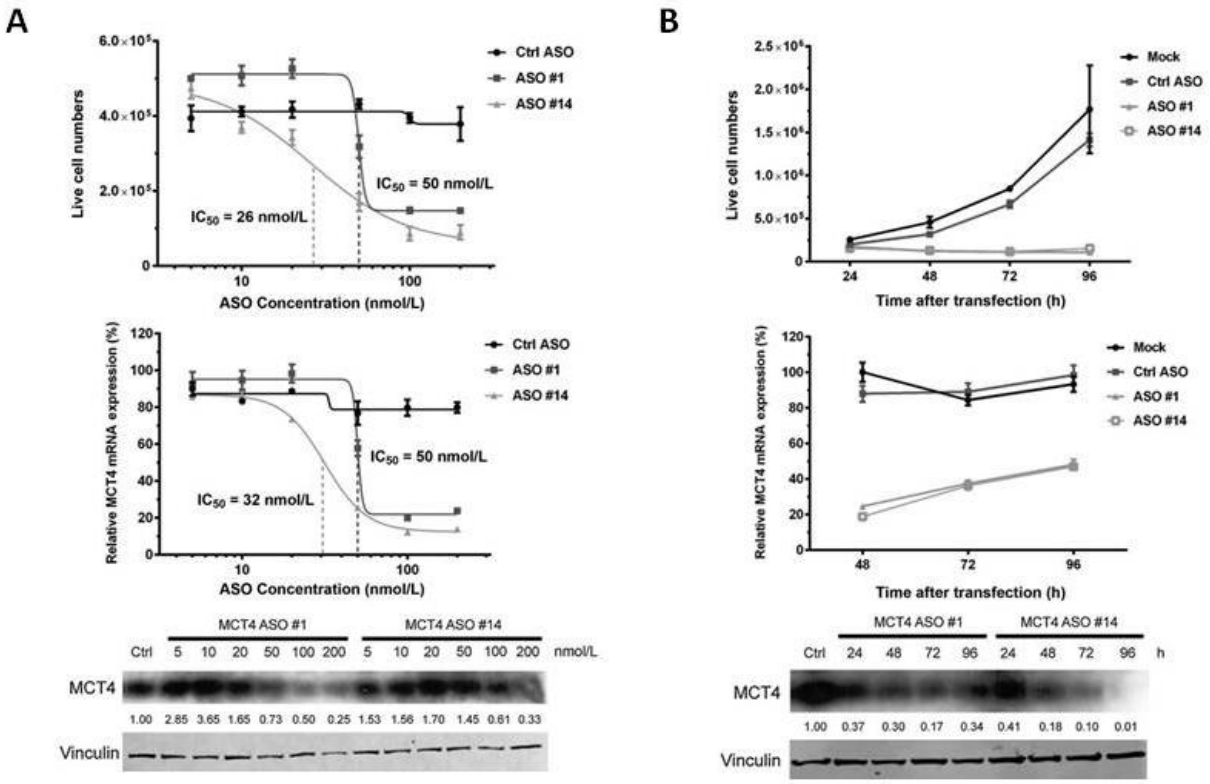

Figure 3.3. Further characterization of MCT4 ASO #1 and #14 reveals a closely mirrored relationship between MCT4 expression and cell proliferation. Transfection of varying amounts of MCT4 ASOs into PC-3 cells demonstrates a close parallel between inhibition of cell proliferation and MCT4 knockdown as measured 48 hours post-transfection. This is evidenced by the near-identical IC_{50} values of each ASO for cell proliferation and MCT4 expression. ASO#1 has an IC_{50} of 50nM for both curves, while ASO#14 has an IC_{50} of 26nM in terms of cell proliferation and 32nM in terms of MCT4 knockdown (A). Furthermore, the sustained inhibition of cell proliferation even up to 96 hours post-transfection also mirrors the sustained reduction of MCT4 expression at an mRNA and protein level (B). Figure reproduced from [382].

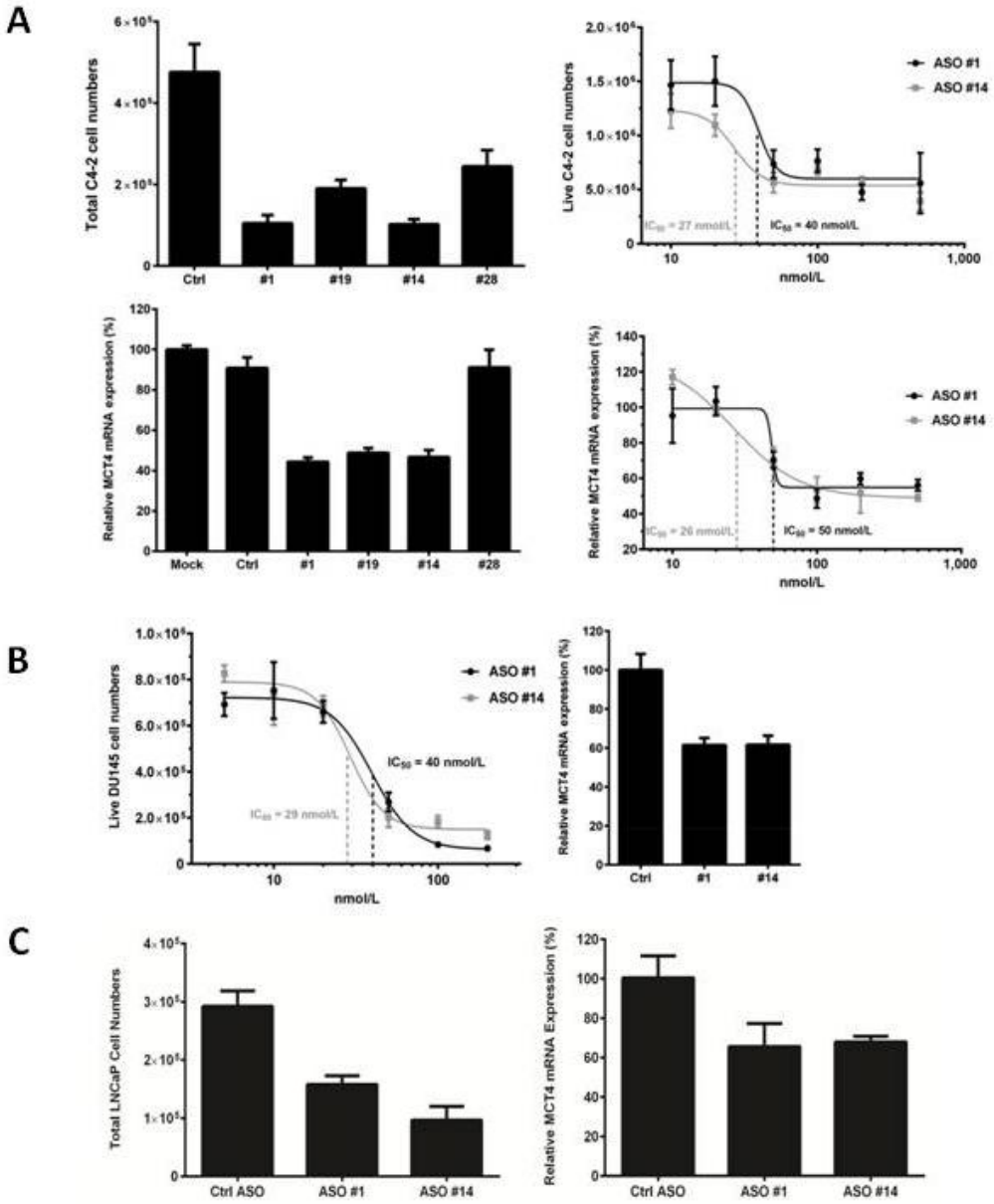

Figure 3.4. MCT4 knockdown in other advanced PCa cell lines also resulted in the inhibition of cell proliferation. Transfection of MCT4 ASOs into the CRPC cell lines C4-2 (A) and DU145 (B) resulted in similar reductions in MCT4 expression and inhibitions of cell proliferation as observed in PC-3 cells 48 hours post-transfection. In particular, comparable IC_{50} values can be observed, ranging from 40-50nM for ASO #1 and 26-29nM for ASO #14 regardless of the parameter measured. Transfection of MCT4 ASO into LNCaP cells also reduced cell proliferation and MCT4 expression at 48 hours (C). Taken together, MCT4 expression appears to be functionally important to the proliferative abilities of glycolytic, advanced PCa cells. Figure reproduced from [382].

MCT4 ASO treatment also resulted in a marked inhibition of lactic acid secretion from PC-3 cells, leading to a corresponding accumulation of intracellular lactate and overall reduction in glucose consumption (Figure 3.5B). A more detailed assessment of changes along the glycolysis and lactic acid production pathways revealed that MCT4 knockdown resulted in a decreased expression of multiple critical genes upstream of lactate secretion. For example, expression of LDHA (a key enzyme involved in converting pyruvate to lactate [293]) and PDK1 (a key kinase regulator involved in switching pyruvate metabolism away from the TCA cycle towards lactic acid production [492]) was found to be downregulated following MCT4 ASO treatment, suggesting a reversion of pyruvate metabolism away from aerobic glycolysis back towards oxidative phosphorylation. Furthermore, other upstream components of the glycolysis pathway, including the glycolytic enzymes GAPDH, PGK1, PGAM1, ENO1, and the glucose transporter GLUT1, were also downregulated following reduction of MCT4 expression (Figure 3.5C).

As such, the results suggest that MCT4 inhibition could have downstream effects on other lactate-associated aspects of cancer biology beyond cell proliferation. Not only can MCT4 inhibition decrease the invasion/migration potential of advanced PCa cells, it can also suppress the elevated glycolytic phenotype, blocking glucose consumption and reducing lactic acid secretion via an overall downregulation of various key genes involved in aerobic glycolysis. As such, the accumulated *in vitro* data suggests that MCT4 inhibition could be an effective therapeutic strategy targeting the fundamental role of cancer-generated lactic acid for treatment of CRPC.

3.3.2.4 Inhibition of MCT4 is Effective Therapeutically against NEPC Cells

The efficacy of MCT4 knockdown was further confirmed in the NCI-H660 NEPC cell line to determine whether similar inhibitions on cell proliferation and glucose metabolism can be achieved. As expected, transfection of MCT4 ASOs #1 and #14 into NCI-H660 cells reduced MCT4 expression without affecting MCT1, MCT2 or CD147 levels, confirming the MCT4-sepcificity of our candidate ASO sequences (Figure 3.6A). Furthermore, similar to previous observations in CRPC cell lines, MCT4

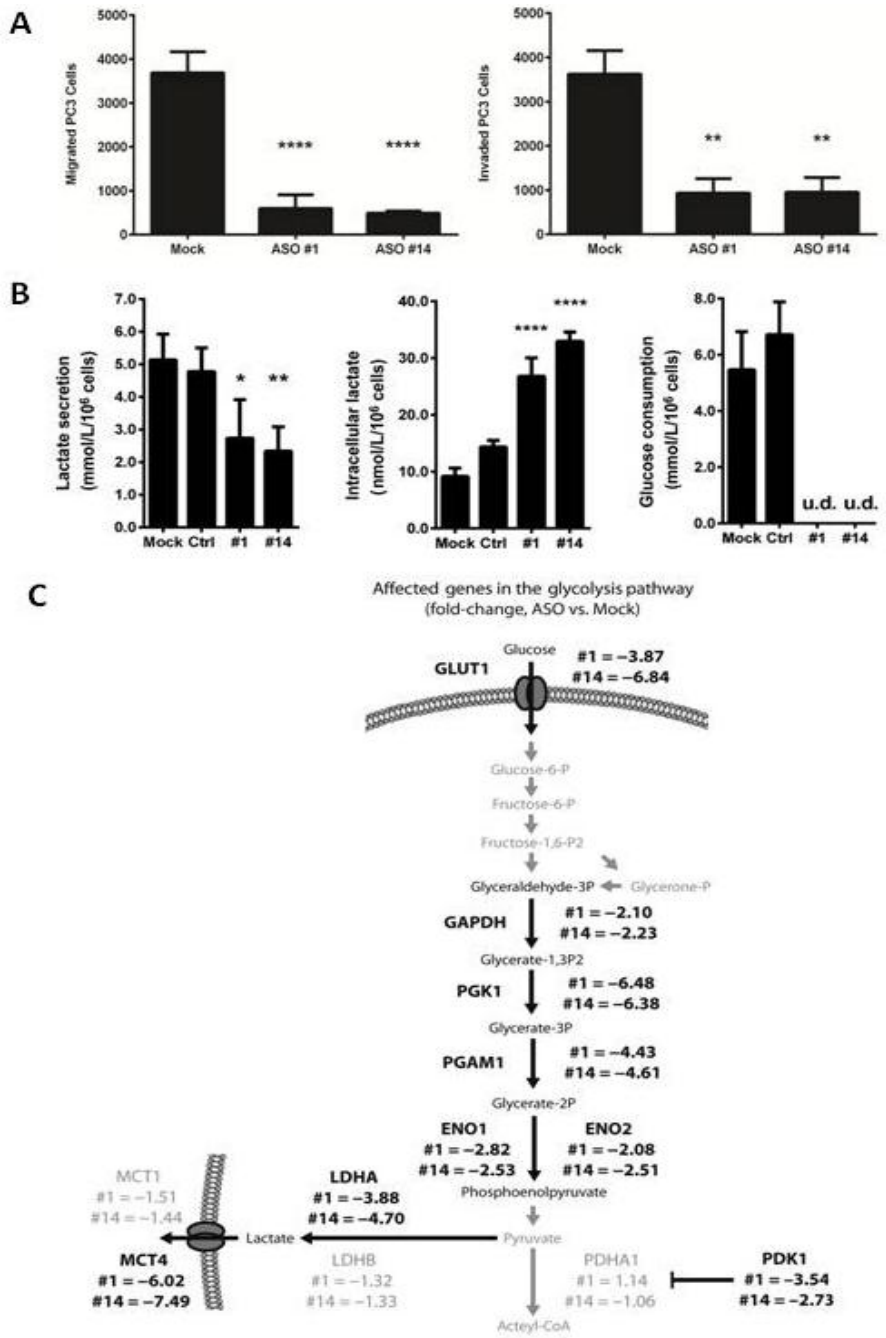

Figure 3.5. MCT4 knockdown decreases invasion/migration potentials and suppresses aerobic glycolysis in PC-3 cells. MCT4 ASO-treated PC-3 cells have reduced transwell migration and invasion capabilities as measured with modified Boyden chambers. The total number of migrated and invaded cells was measured by fluorescence spectroscopy after 48 hours of incubation (A). Furthermore, MCT4 ASO treatment reduced lactic acid secretion, increased intracellular lactate accumulation, and inhibited glucose consumption as determined by the respective colorimetric assays (B). A downregulation of multiple upstream genes in the glycolysis and lactic acid production pathways was also observed by qPCR. Affected genes are highlighted with fold-changes after ASO treatment indicated numerically (C). Statistical analyses were carried out using one-way ANOVA with post-hoc Dunnett's tests; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ud, undetectable. Figure reproduced from [382].

knockdown was able to inhibit NEPC cell proliferation (Figure 3.6B). This inhibition of cell proliferation was concomitant with a suppression of lactic acid secretion and reduction in glucose consumption (Figure 3.6C), suggesting that MCT4 is functionally important in facilitating proliferation and glucose metabolism in NEPC cells as well.

More significantly, decreased expressions of various genes in the glycolysis and lactic acid production pathways were also observed in NEPC cells following MCT4 ASO treatment, comparable to previously described changes in CRPC. These downregulated genes include the aforementioned enzymes and modulators key to regulating aerobic glycolysis such as LDHA, PDK1, ENO1, PGK1, and GLUT1 (Figure 3.6D). The close parallel between changes observed in glycolytic CRPC and NEPC cell lines following MCT4 knockdown suggests a common mechanism of action. Inhibition of MCT4-mediated lactic acid secretion could result in the accumulation of intracellular lactate, reducing cell proliferation and causing a feedback inhibition of upstream glucose metabolism via downregulation of genes in the glycolysis and lactic acid production pathways. Furthermore, the biological consequences of MCT4 knockdown in NEPC cells provide functional evidence for our earlier metabolic pathway analyses, confirming that elevated glycolysis and increased lactic acid production/secretion is indeed a relevant metabolic phenotype to NEPC tumours.

Taken together, MCT4 inhibition appears to be efficacious against advanced PCa cells. In particular, as both CRPC and NEPC cells seem to rely on MCT4 expression and function for proliferation, invasion/migration, and glucose metabolism, treatment using MCT4 ASOs could potentially be an effective therapeutic strategy for the clinical management of aggressive CRPC and NEPC.

3.3.3 Efficacy of MCT4 Inhibition *in vivo*

MCT4 appears to be a promising therapeutic target for treatment of CRPC and NEPC given the initial *in vitro* results. Effective and specific MCT4-taregeting ASOs were identified, and reduction of MCT4 expression inhibited cell proliferation across multiple CRPC and NEPC cells lines. Furthermore, MCT4 expression appears to be functionally important in facilitating invasion and migration

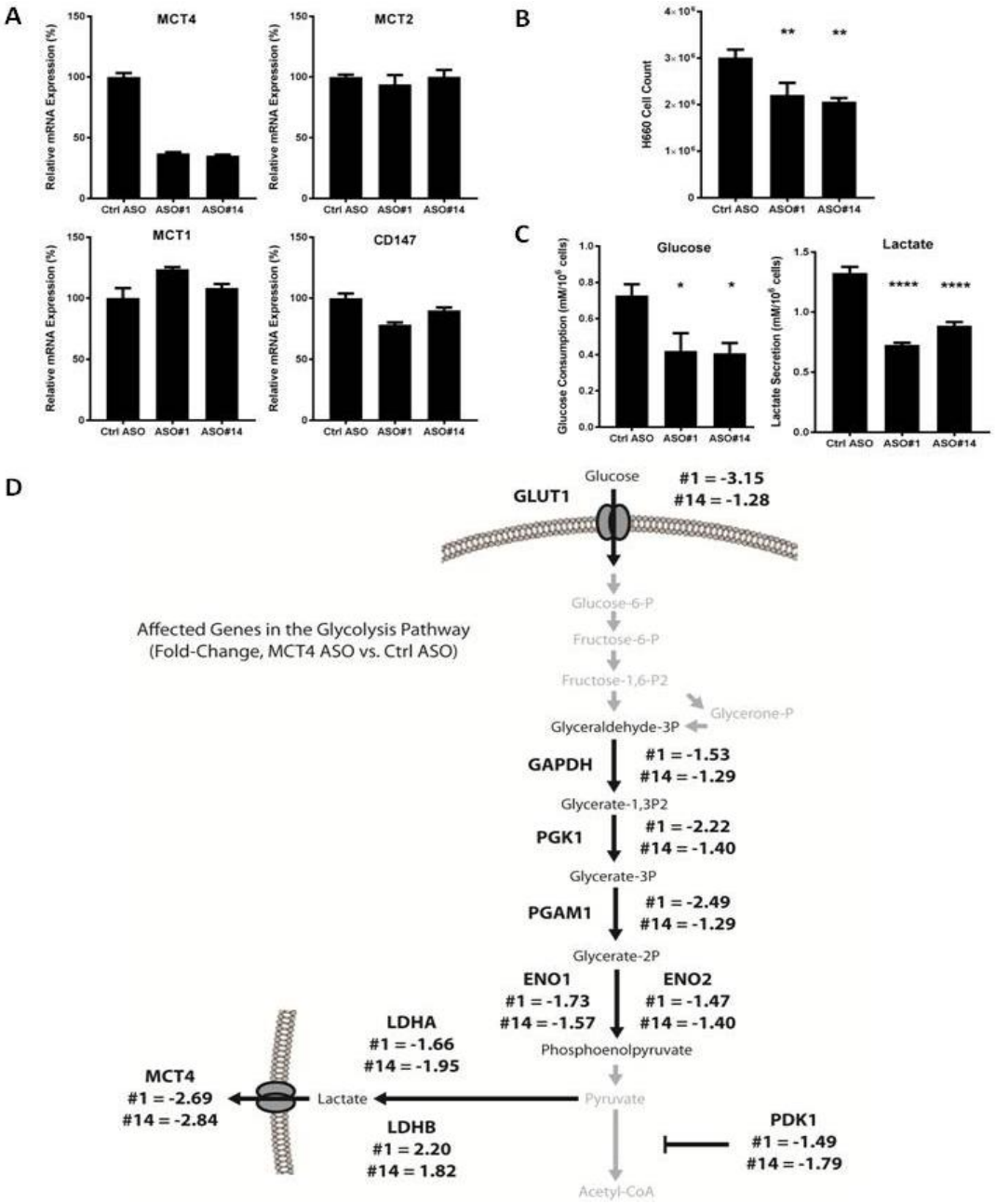

Figure 3.6. MCT4 knockdown in the NCI-H660 NEPC cell line was also effective at decreasing cell proliferation and inhibiting glucose metabolism. MCT4 ASO treatment in NCI-H660 cells resulted in decreased MCT4 expression without affecting the levels of other MCT family members as measured by qPCR 96 hours post-transfection (A). Furthermore, decreased MCT4 expression resulted in reduced cell proliferation as measured by live cell counts (B) and reduced lactate secretion and glucose consumption as measured by the respective colorimetric assays (C). MCT4 knockdown also inhibited upstream glycolysis as measured by qPCR in a similar fashion to PC-3 cells, suggesting that reduced expression of multiple key genes in the glycolysis and lactic acid production pathways could be a common mechanism of action. Affected genes are highlighted with fold-changes after ASO treatment indicated numerically (D). Statistical analyses were carried out using one-way ANOVA with post-hoc Dunnett's tests; *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

in advanced PCa. More importantly, inhibition of MCT4 also resulted in reduced lactate secretion and glucose consumption. This inhibition of glucose metabolism following MCT4 knockdown is observed in both CRPC and NEPC cells, where multiple upstream enzymes and regulators critical to aerobic glycolysis were downregulated as a key mechanism of action. As such, we proceeded to assess the treatment efficacy of MCT4 ASOs *in vivo*. Based on our hypothesis, we suspect that in addition to promoting tumour growth directly, MCT4 may also facilitate the establishment of an immunosuppressive acidic tumour microenvironment as a result of excessive cancer-generated lactic acid secretion. We therefore selected two *in vivo* systems that would allow for a preliminary assessment of anticancer immunity while evaluating the treatment efficacy of MCT4 inhibition against human PCa.

3.3.3.1 MCT4 ASO Treatment Inhibits PC-3 Tumour Growth in Nude Mice

PC-3 cells were subcutaneously transplanted into both flanks of male athymic nude mice for treatment with candidate MCT4 ASOs #1 and #14. Intraperitoneal injections of ASOs at 10mg/kg for 15 days did not induce major host toxicity, as indicated by the fact that animal weights in all groups remained stable throughout the treatment duration (Figure 3.7A) without observable abnormal host behaviours. Both MCT4 ASOs were able to reduce the growth of PC-3 tumours when compared to the vehicle control or the ASO control using a non-targeting sequence (Figure 3.7B), suggesting that MCT4 inhibition can be an effective therapeutic strategy for treatment of advanced PCa. Further analysis of the treated tumours by IHC revealed that MCT4 ASOs induced greater tumour cell apoptosis and decreased tumour cell proliferation, as measured by positive cleaved caspase-3 staining and positive Ki-67 staining respectively (Figure 3.7C). Importantly, the reduction in tumour growth was also associated with decreased MCT4 expression in MCT4 ASO-treated tumours (Figure 3.7D). This is consistent with *in vitro* observations that MCT4 knockdown results in a reduction of cell proliferation via inhibition of glucose metabolism.

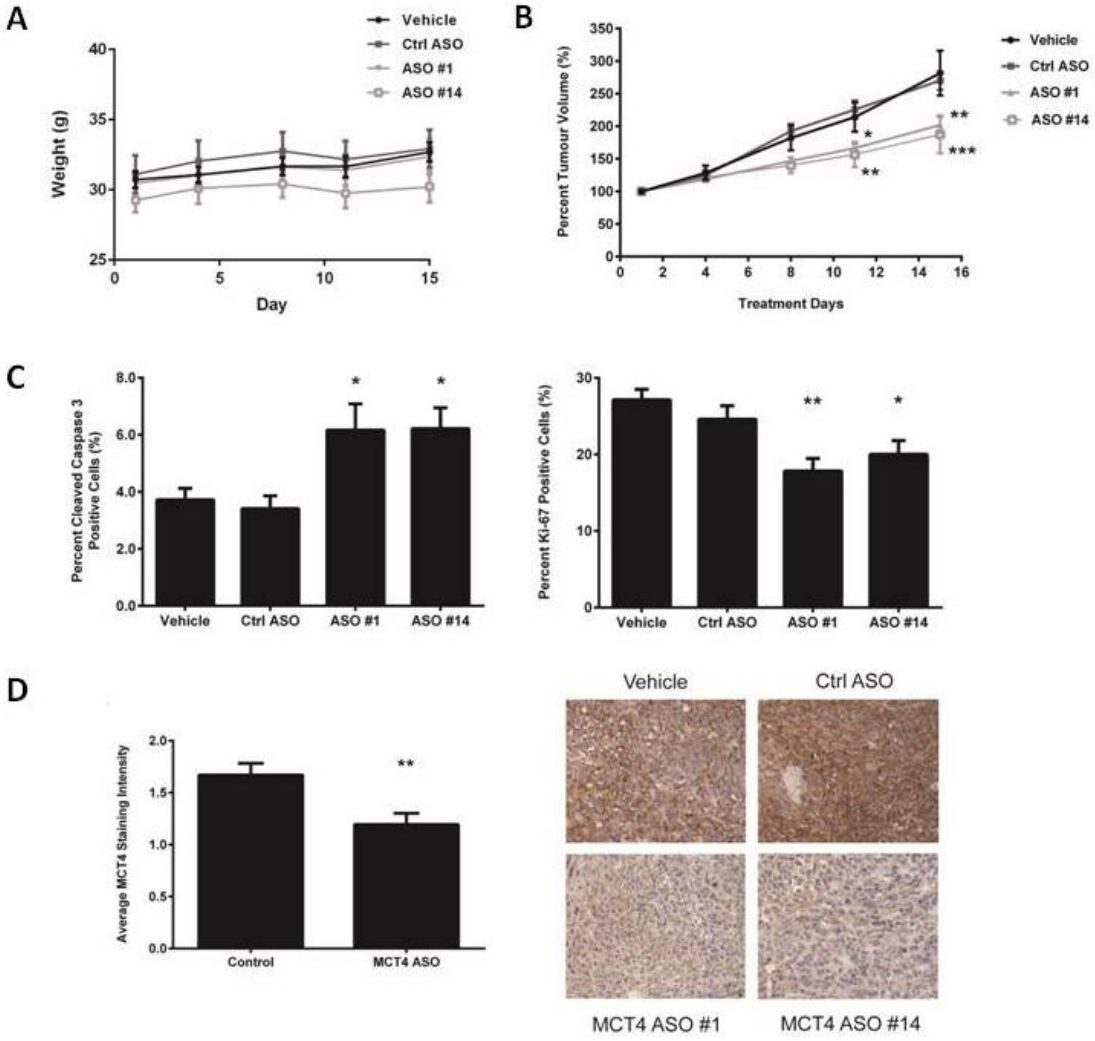

Figure 3.7. MCT4 ASO treatment shows *in vivo* efficacy in PC-3 tumour-bearing nude mice. Two weeks' treatment by daily i.p. injections of MCT4 ASOs at 10mg/kg did not induce major host toxicity as evidenced by stable host body weights throughout the duration of treatment (A). More importantly, MCT4 ASO treatment reduced subcutaneous PC-3 tumour growth as measured by tumour volume. Statistical analysis was done using two-way ANOVA with post-hoc multiple comparison; *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$ (B). Increased tumour cell apoptosis and reduced tumour cell proliferation was also observed by IHC staining, as indicated by increased percentages of cleaved-caspase 3-positive and reduced percentages of Ki67-positive tumour cells respectively following MCT4 ASO treatment (C). This reduction in PC-3 tumour growth following MCT4 ASO treatment is observed in conjunction with decreased MCT4 expression as measured by IHC staining (D). This is consistent with *in vitro* observations that MCT4 knockdown reduces cell proliferation and inhibits glucose metabolism. Statistical analyses were done using one-way ANOVA with post-hoc Dunnett's tests; *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$. Figure reproduced from [382].

3.3.3.2 MCT4 ASO Treatment Potentially Enhances Anticancer Immunity in Nude Mice

Given our hypothesis that excessive cancer-generated lactic acid can induce an acidic immunosuppressive tumour microenvironment [343], we investigated whether MCT4 ASO treatment in PC-3 tumour-bearing nude mice altered the local host immune response. Although immunodeficient hosts are required for the study of human cancers *in vivo*, certain aspects of innate immunity remain intact in nude mice [493-495]. As such, some initial, albeit limited, insights into the immunomodulatory effects of MCT4 inhibition may still be gleaned.

Additional IHC staining assessing the effects of MCT4 ASO treatment on host immunity was performed. Interestingly, a staining of endothelial cells using CD31 revealed that a significant number of immune cells have extravasated from and aggregate around tumour-associated blood vessels. This phenomenon is particularly evident surrounding blood vessels in the tumour periphery. A quantitative comparison of the most prominent areas of immune cell aggregation shows that tumours treated with MCT4 ASOs had significantly larger immune cell aggregates than control-treated tumours (Figure 3.8A). Furthermore, as NK cells are the predominant functional cytotoxic immune cells in nude mice [496], an assessment of their presence was done by NK1.1 staining. Quantification of positive staining in the same prominent immune cell aggregates revealed that the proportion of tumour-associated NK cells was significantly increased in MCT4 ASO-treated tumours (Figure 3.8B). Finally, although CD3 is commonly associated with the T-cell receptor complex and thus regarded as a T-cell marker, its expression and function can also facilitate activation of NK cell cytotoxicity [497, 498]. As nude mice lack functionally mature T cells, CD3 staining can indicate NK cell activation instead [499]. We found that the proportion of activated NK cells associated with ASO-treated tumours is also increased compared to control tumours (Figure 3.8C). Taken together, the results provide initial evidence supporting our hypothesis that MCT4-mediated lactic acid secretion induces an acidic immunosuppressive tumour microenvironment.

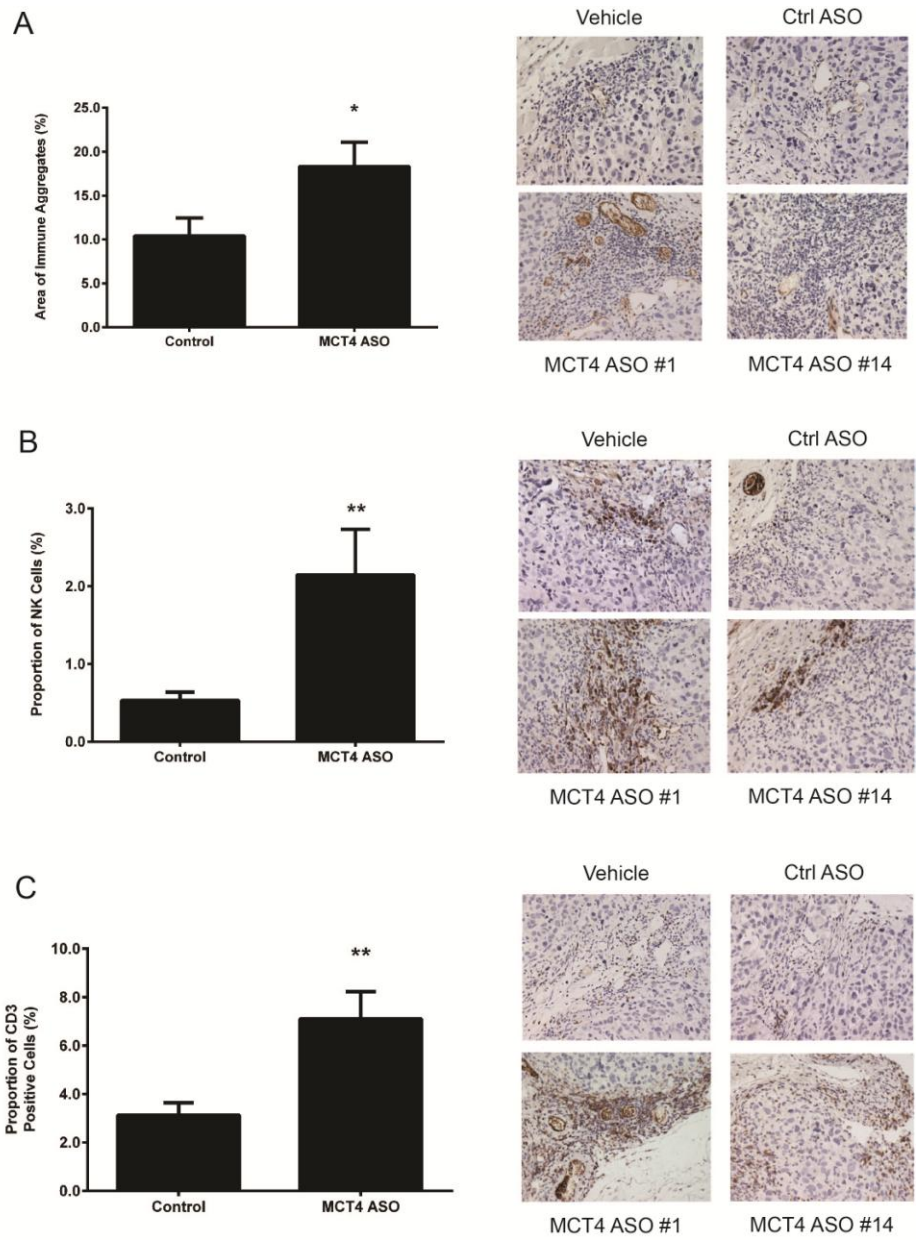

Figure 3.8. MCT4 ASO treatment potentially enhances anticancer immunity by increasing NK cell proportions in PC-3 tumours. MCT4 ASOs can potentially have immunomodulatory effects *in vivo* as treatment increased the extent of immune cell extravation and aggregation in the tumour periphery. This is observable by IHC staining as the increased accumulation of cells with small, round nuclei (A). Characterization of the immune cells present revealed that there is an increase in the proportion of NK cells as assessed by IHC staining of NK1.1 (B). These NK cells have also been activated following MCT4 ASO treatment, as evidenced by positive CD3 staining (C). These observations provide an initial indication that MCT4 may indeed play an important role in inducing an acidic immunosuppressive tumour microenvironment. Statistical analyses were done using student's t-tests; *, $p < 0.05$; **, $p < 0.01$. Figure reproduced from [382].

3.3.3.3 MCT4 ASO Treatment in First-generation PDXs Stimulate Patient Immune Cells

Given the challenges associated with investigating anticancer immunity in xenograft contexts, a second model system involving first-generation PDXs was used to confirm our findings in PC-3 tumour-bearing nude mice. We have previously developed this unique methodology based on our observations that patient tumour-associated stromal tissue remains viable and functional during the first three months of grafting and is only later replaced by mouse stromal components after serial transplantation [500]. As such, patient tumour-associated immune cells are still present within the PDX tissue and remain functional during the early days following engraftment. This allows for changes in patient immune-cancer cell interactions to be assessed in a relatively native environment after experimental therapeutic interventions, uniquely supplementing traditional efficacy data and potentially revealing synergistic effects following reactivation of anticancer immunity.

Similar to observations in PC-3 tumour-bearing nude mice, treatment of first-generation PDXs using MCT4 ASO #14 was able to reduce PCa tumour viability (Figure 3.9A). This further confirms that MCT4 inhibition can be an effective therapeutic strategy. More importantly, IHC staining of first-generation PCa PDX tumours using human-specific antibodies revealed that MCT4 ASO #14 treatment can potentially elicit an anticancer immune response from tumour-associated immune cells, a phenomenon absent from control ASO-treated tumours. More specifically, a drastic increase in proliferative patient immune cells was observed following MCT4 ASO treatment, as evidenced by an increased abundance of small, round, human Ki-67 positive cells (Figure 3.9B). Further characterization of these tumour-associated immune cells revealed that MCT4 ASO #14-treated tumours had significantly more CD45-positive human lymphocytes (Figure 3.9C). A corresponding increase in human CD8-positive immune cells was also observed (Figure 3.9D), suggesting that these lymphocytes are of a CD8 cytotoxic T-cell lineage. These changes are evident even from representative images of the IHC analysis (Figure 3.9E). As such, MCT4 ASO treatment appears to be able to stimulate the proliferation of tumour-associated immune cells and promote their differentiation into the CD8 cytotoxic T cells, potentially facilitating an enhanced MCT4 inhibition-mediated anticancer immunity.

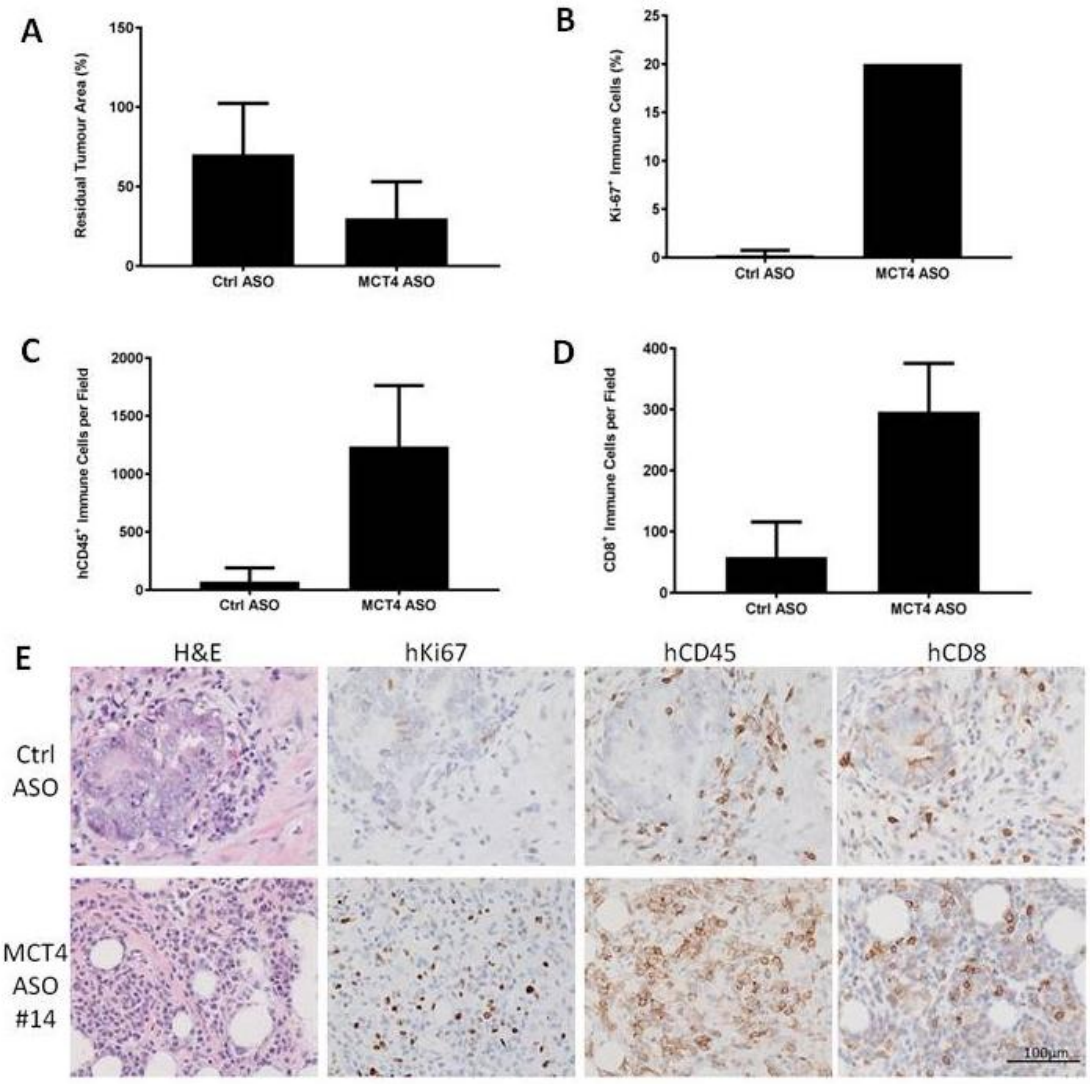

Figure 3.9. MCT4 ASO treatment stimulate patient immune cell proliferation and differentiation in a first-generation PCa PDX model. Treatment with MCT4 ASO #14 reduced first-generation PCa PDX tumour viability. This was calculated by assessing the proportion of residual live tumour tissue as a percentage of the entire graft after H&E staining and reflects the *in vivo* treatment efficacy previously observed (A). Furthermore, MCT4 ASO treatment induced patient tumour-associated immune cell proliferation as assessed by IHC staining of Ki-67 (B). This ultimately increases the number of tumour-associated CD45-positive human lymphocytes (C). A concurrent increase in human CD8-positive cytotoxic T cells was also observed (D). These changes to the local immune cell population is reflected in representative images of IHC staining (E). As such, effective MCT4 inhibition as induced by MCT4 ASOs could reverse the immunosuppressive effects of the tumour microenvironment and stimulate anticancer immunity.

3.4 Discussion

We have previously proposed that altered cancer metabolism and cancer-generated lactic acid can promote tumour growth through a number of downstream mechanisms [343]. In particular, the role of MCT4-mediated lactic acid secretion seemed particularly relevant to assisting cancer cell proliferation, inducing invasion/metastasis, and maintaining a localized acidic immunosuppressive tumour microenvironment. Here, we were able to functionally verify MCT4 as a key mediator of lactic acid secretion and an essential facilitator of multiple lactate-associated downstream processes. Furthermore, elevated MCT4 expression was confirmed as clinically relevant to advanced PCa patients. As such, MCT4 is a promising therapeutic target of critical functional importance. A therapeutic strategy focused on inhibiting MCT4 action could thus be potentially effective for the clinical management of CRPC and NEPC, as well as other glycolytic tumour types.

As a proof-of-concept study investigating the biological function of MCT4 and the potential therapeutic effects of its inhibition, a panel of MCT4 ASOs were designed and verified to be both effective at decreasing MCT4 expression and specific to human MCT4. MCT4 knockdown in various advanced PCa cell lines resulted in consistent suppression of cell proliferation, reduction in lactic acid secretion, and inhibition of glucose consumption. Importantly, MCT4 knockdown also induces an upstream downregulation of genes in the glycolysis and lactic acid production pathways. A decrease in LDHA and PDK1 expression following MCT4 ASO transfection suggests a shift in pyruvate metabolism away from lactic acid production towards utilization in the TCA cycle. Additionally, a downregulation of GLUT1 together with other glycolytic enzymes following MCT4 knockdown also suggests a general reduction in glucose metabolism consistent with reduced lactic acid secretion. Since this phenomenon is observed in both CRPC and NEPC cells, it could be a key common mechanism of action following therapeutic inhibition of MCT4. Not insignificantly, MCT4 knockdown also reduced the invasion and migratory potentials of PC-3 cells, suggesting that MCT4 action can indeed promote additional downstream lactate-associate processes as proposed by our hypothesis.

The therapeutic efficacy of MCT4 inhibition was further confirmed *in vivo* using our candidate MCT4 ASOs. As our hypothesis regarding the tumour-promoting functions of MCT4 includes a potential suppression of the anticancer immune response, two xenograft models with some functional remnants of immunity were used. PC3 tumour-bearing nude mice lack mature T cells but have an active cytotoxic NK cell population [496]. First-generation PCa PDXs lack systemic functional host immunity but have viable patient tumour-associated immune cells [500]. As such, these *in vivo* model systems balance the ability to facilitate human PCa growth for assesssing treatment efficacy with the ability to elucidate the potential immunomodulatory effects following MCT4 inhibition. Similar to observations *in vitro*, MCT4 ASO treatment was able to reduce PCa tumour growth *in vivo*. More remarkably, MCT4 ASO treatment showed potential signs of stimulating anticancer immunity in both model systems. In PC3 tumour-bearing nude mice, MCT4 ASO-treated tumours had larger extravasated immune cell aggregates and greater tumour-associated activated NK cell proportions. In first-generation PCa PDXs, treatment with MCT4 ASO#14 induced the proliferation of patient tumour-associated immune cells and increased the presence of human CD8-positive cytotoxic T cells. As such, not only were we able to confirm the efficacy of MCT4 ASOs at reducing tumour growth *in vivo*, we were also able to demonstrate that MCT4 inhibition could lead to an enhancement and stimulation of anticancer immunity.

Overall, results from our *in vitro* studies support our hypothesis that MCT4-mediated lactic acid secretion promotes the cancer hallmark characteristics of altered metabolism, sustained cell proliferation, and invasion/metasatsis [343, 501]. Results from our *in vivo* studies further confirm the role of MCT4 in supporting tumour growth. More importantly, MCT4 inhibition seems to reverse the immunosuppressive effects of the tumour microenvironment, with ASO treatment potentially stimulating and enhancing the anticancer immune response. As such, we provide evidence to additionally implicate MCT4 action in promoting the cancer hallmark characteristic of avoiding immune destruction [501]. Although we did not experimentally verify here in this study the effects of MCT4-mediated lactic acid secretion on other cancer-promoting properties such as angiogenesis and response to hypoxia, it is not unimaginable that

these processes could also be facilitated by MCT4 action, especially given its involvement in the four major aforementioned cancer hallmarks.

Taken together, our results offer experimental confirmation in support of our hypothesis that cancer-generated lactic acid plays a central tumour-promoting and immunosuppressive role [343] with key contributions from MCT4 action. Additionally, our results suggest that a single-target agent inhibiting MCT4 function could have combinatorial therapeutic impacts on multiple crucial cancer characteristics. Furthermore, an even greater treatment response may be observed in a clinical setting, where the full complement of a patient's anticancer immune response may become reactivated following the reversal of an acidic tumour microenvironment. Given the near-universal phenomenon of altered cancer metabolism and the wide-ranging effects of cancer-generated lactic acid, effective MCT4 inhibition could be a broadly applicable therapeutic strategy for treatment of multiple malignancies.

While first-generation ASOs were employed in this current study for proof-of-concept purposes, ASOs with generation 2.5 base modifications are a *bona fide* therapeutic modality with multiple FDA approvals and ongoing clinical trials [502-504]. In view of the real possibilities for future commercialization and clinical application, the 23 effective MCT4-targeting ASO sequences have been submitted for patent protection. Ongoing efforts are currently underway to confirm the *in vitro* and *in vivo* efficacy of generation 2.5 MCT4 ASOs. Additionally, investigations exploring the potential of these ASOs for therapeutic use in other malignancies could expand the clinical space beyond advanced PCa. As such, the findings detailed in this study have broad implications for the clinical management of cancer patients, both in terms of outlining a novel perspective for understanding cancer biology and offering a potentially effective therapeutic strategy ready for the more rigorous validations necessary for assessing clinical utility.

Chapter 4: Development of MCT4 Small Molecule Inhibitors (SMIs)

4.1 Introduction

Small molecule inhibitors (SMIs) have long been the standard default therapeutic entity across a number of different diseases, with new chemical compounds comprising approximately 83% of FDA approvals in the past two decades [505]. In the cancer context, development of chemical inhibitors have progressed from broad cytotoxic chemotherapeutics to more select inhibitors aimed at attenuating oncogenic functions of specific protein targets [402, 506]. Protein kinases remain the predominant class of therapeutic targets for SMI development in cancer therapy [507]. This can be attributed to their relatively well-studied and druggable ATP-binding pockets as well as the early clinical successes of BCR-ABL and EGFR inhibitors [508]. However, recent advances have also been made in developing small molecules targeting other protein classes, including those transcription factors or mediators of protein-protein interactions previously described as difficult-to-target [402, 509, 510]. This suggests that a broad category of cancer-promoting proteins may become increasingly amenable to interventions with SMIs.

Having demonstrated in the proof-of-concept study that MCT4 inhibition can be an effective therapeutic strategy for treatment of advanced PCa by affecting multiple downstream lactate-associated tumour-promoting processes, we proceeded to consider in greater detail potential MCT4 inhibition strategies for clinical application. Pharmaceutical development has notoriously high attrition rates, with the latest statistics suggesting that only 8% of positive preclinical results translate into successful phase I clinical trials [216]. Furthermore, figures for all disease indications from the past decade show that only 9.6% of phase I clinical trials attain ultimate regulatory approval, with the probability dropping to 5.1% when considering only oncology applications [511, 512]. As such, investigating multiple therapeutic modalities in parallel, especially in the preclinical stages of development, could be a potentially beneficial strategy for identifying a successful clinical candidate. The use of biologics for therapeutic purposes has recently gained momentum and is becoming a larger percentage of treatments entering the clinic, comprising 32% of approved entities in 2016 [505]. Although ASOs fall within the biologics category, the majority of biomacromolecules under clinical investigation remain therapeutic antibodies.

Unfortunately, MCT4 is a channel protein with relatively small extracellular domains (the largest two being only 21 and 14 amino acids in length [513]), making inhibitory antibody development potentially challenging. However, as MCT4 primarily functions as a transporter of soluble metabolic small molecules, a drug-binding pocket is likely already naturally present. Therefore, efforts into developing potent and specific MCT4-targeting SMIs appear comparatively more promising with greater likelihood of success.

SMI development strategies have historically fallen along a conceptual spectrum with randomized compound screening on the one hand and rational drug design on the other. As the possible chemical space for low molecular weight compounds reaches upwards of 10^{40} to 10^{100} unique entities, even a drastically smaller subspace of potentially therapeutically active molecules is estimated at over 24 million compounds [514]. As such, chemical libraries containing millions of drug-like molecules are often assessed by validated assays in a robotically-assisted, high-throughput manner to identify potential hits with therapeutic effects in a randomized, unbiased manner [515]. Unfortunately, such traditional high-throughput drug screening strategies require extensive investments from a financial, experimental, and labour perspective and are not routine approaches to many research endeavours. Alternatively, if sufficient information is available regarding a therapeutic target of interest, especially from a structural biology and ligand-binding perspective, it may be possible to predict in a knowledge-based manner potential inhibitors and experimentally assess only 10-1000 compounds [402]. However, as many therapeutic targets remain relatively novel, the lack of accurate information regarding structure and function may make rational drug design untenable. Consequently, the development of effective therapeutic compounds remains a major hurdle in translational research, contributing substantially to the gap between laboratory discoveries and clinical applications.

Recent advances in computational technology have offered a promising path to bridging the two sides of the drug development spectrum, bringing greater efficiencies and cost-reductions to identifying potentially effective therapeutic SMIs. In particular, applications of big data analytics [516], GPU-computing [517], and deep learning [518] to model drug binding and potential therapeutic responses have

propelled drug discovery into a new era employing *in silico* methodologies [519, 520]. For example, while additional emerging technologies such as cryo-electron microscopy (cryo-EM) can help determine the structure of previously uncrystallized proteins at resolutions comparable to traditional X-ray crystallography [521, 522], potential 3D structures can also be accurately modelled using sophisticated structural prediction algorithms, thereby identifying possible drug binding pockets to assess the goodness of small molecule fitting [523]. Furthermore, substructure pattern recognition by machine learning can also help predict the absorption, distribution, metabolism, excretion and toxicity (ADMET) characteristics of compounds, thereby eliminating potentially toxic drug candidates early in the discovery process [524, 525]. Importantly, the use of computational modelling can mimic the randomized drug screening process, virtually docking millions of compounds [526] to a therapeutic target of interest and identifying potential hits for experimental validation. As such, the use of an *in silico* drug discovery pipeline can achieve the results expected of an unbiased high-throughput screen with resources on a rational drug design scale.

The VPC has established a track record of applying such computer-aided drug discovery methodology to successfully develop SMIs at protein-protein and protein-DNA interaction sites of critical drug targets in cancers, including AR [527-529], estrogen receptor [530], and ERG [531]. In collaboration with Dr. Artem Cherkasov, we employed a similar strategy to identify potential MCT4 SMIs for experimental evaluation. Initial hit compounds were identified and their chemical analogues were rescreened as a first round of hit optimization. Our efforts constitute the first steps towards ultimately developing potent and specific MCT4 SMIs for the clinical application of an MCT4 inhibition therapeutic strategy in treatment of advanced PCa and beyond.

4.2 Materials and Methods

All materials and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated.

4.2.1 Human MCT4 Modeling, Virtual Compound Screening, and *in silico* Hit Optimization

Computer-assisted drug screening was done in collaboration with Dr. Artem Cheraksov and his post-doctoral fellow Dr. Michael Hsing using the in-house *in silico* pipeline established at the VPC. The overall workflow has been previously published and extensively described elsewhere [527, 528, 531]. In brief, 3D computer models of human MCT4 were first constructed with the I-TASSER computational protein threading program [523], using the crystal structures of *Escherichia coli* glycerol-3-phosphate transporter (PDB: 1PW4) and lactose permease (PDB: 1PV6) as templates. The predicted structural models were then compared to a published MCT1 model [532] and subjected to rigorous molecular dynamics (MD) simulations with the phospholipid bilayer module implemented in the Desmond Molecular Dynamics System from Schrödinger Suites (Schrödinger, LLC., New York, NY, USA). Stable MCT4 MD conformations were then used to identify drug-binding pockets by virtual atomic probing using the Molecular Operating Environment (MOE) program from Chemical Computing Group (Montreal, QC, Canada).

A total of four million small molecules were extracted from the ZINC database [526] for virtual compound screening. After protonation and energy minimization, each small molecule was docked at the top-ranked drug-binding pocket from our human MCT4 model using multiple molecular docking programs including Glide [533], FRED [534], and eHiTS [535]. A consensus voting method was used to select the top virtual hits, taking into account docking scores, predicted binding affinities, consistency among docking poses, and drug-like features described by Lipinski's rule of five [536]. The final list of candidate molecules was visually evaluated, verifying favourable interactions with nearby protein residues, desirable occupancy in the binding pocket, and lack of problematic or promiscuous moiety. Favourable predicted ADMET properties were assessed by FAFDrugs [537] and the ADMET Predictor module from SimulationsPlus (Lancaster, CA, USA).

Initial hit compounds validated by experimental assays were subjected to first-round *in silico* optimizations. Chemical similarity searches, pharmacophore modeling, MD simulations, and free energy perturbation (FEP) assessments [538] were used to model quantitative structure-activity relationships

(QSAR) [539]. A list of chemical analogues related to the initial hit compounds was generated for a second round of experimental evaluation.

4.2.2 Preparation of Stock Solutions

Compounds identified through the virtual drug screening and *in silico* first-round optimization were purchased from Vitas-M Laboratory (Champaign, IL, USA), ChemDiv (San Diego, CA, USA), Enamine (Kiev, Ukraine), ChemBridge (San Diego, CA, USA), or Princeton BioMolecular Research (Monmouth Junction, NJ, USA) based on supplier availabilities. Chemicals were obtained at a 5mg quantity (or as available) and dissolved in the appropriate volume of DMSO for a stock concentration of 50mM.

4.2.3 Cell Cultures

The human glioblastoma cell line U-251 MG was purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, United Kingdom) and maintained in DMEM (GE Healthcare HyClone) supplemented with 10% FBS (GE Healthcare HyClone). The human osteosarcoma cell line U-2 OS and human CRPC cell line PC-3 were purchased from the ATCC and maintained in RPMI-1640 (GE Healthcare HyClone) supplemented with 10% FBS. The human renal carcinoma cell lines A-498 and 786-O were also purchased from the ATCC and were maintained in DMEM supplemented with 10% FBS. The human CRPC cell line C4-2 was obtained from Dr. Martin E. Gleave (VPC) and maintained in RMPI-1640 supplemented with 10% FBS.

4.2.4 Initial Evaluation of Therapeutic Efficacy

An initial single-dose assessment of therapeutic efficacy was done at 50uM for all purchased compounds. Alterations to cell proliferation in A-498, 786-O, and U-2 OS cells at 144 hours after treatment were assessed by MTS using the CellTiter 96 AQueous One Solution Reagent from Promega (Madison, WI, USA). Changes to cell proliferation 72 hours after treatment in U-251 MG cells were

assessed by live cell counts using the TC20 automated cell counter with trypan blue (BioRad). Potential therapeutic efficacy in U-251 MG cells were further confirmed by visual assessments. Changes to cell morphology were noted after observations under the microscope, while shifts in culture media acidity were marked by colour change from red (physiological pH) to yellow (acidic pH). The treatment effects of candidate compounds were compared to observations following MCT1 or MCT4 siRNA (Dharmacon) reverse transfection using Lipofectamine RNAiMAX (Invitrogen). Treatment using the nanomolar MCT1/2 inhibitor AZD3965 (SelleckChem, Houston, TX, USA) was also used as control.

Potentially effective hit compounds were further characterized by dose dependence analyses to compare their potency. IC_{50} values were calculated using the GraphPad Prism 6 statistical analysis software. Changes to glucose metabolism following treatment with the effective hit compounds were also assessed by measuring glucose consumption and lactic acid secretion using colorimetric assays from BioVision, Inc. as described in Section 3.2.9.

4.3 Results

4.3.1 Human MCT4 Modeling Reveals a Potential Intra-Channel Drug-Binding Pocket

Despite the current lack of available crystal structures for any mammalian MCT family members, we were able to apply computational protein threading methods to build multiple 3D models based on structural templates from other related transporters (Figure 4.1A). A comparison of these predicted structures suggests that MCT4 model #2 is the most similar and bears the greatest resemblance to a previously published mouse MCT1 structural model prediction [532]. Structural superimposition resulted in a moderate root-mean-square deviation (RMSD) of 5Å. This can primarily be attributed to protein sequence variations, wherein human MCT4 and human MCT1 share only 47% protein sequence identity (Figure 4.1B). Also similar to findings from the published MCT1 model is the potential MCT4 drug-binding site. Earlier assessments of MCT1 indicated that specific inhibitors bound to the protein from within the channel in an inward-open resting conformation [532, 540]. Analysis of the inward-open MCT4 model #2 using virtual atomic probes and MOE software have also suggested that the potential

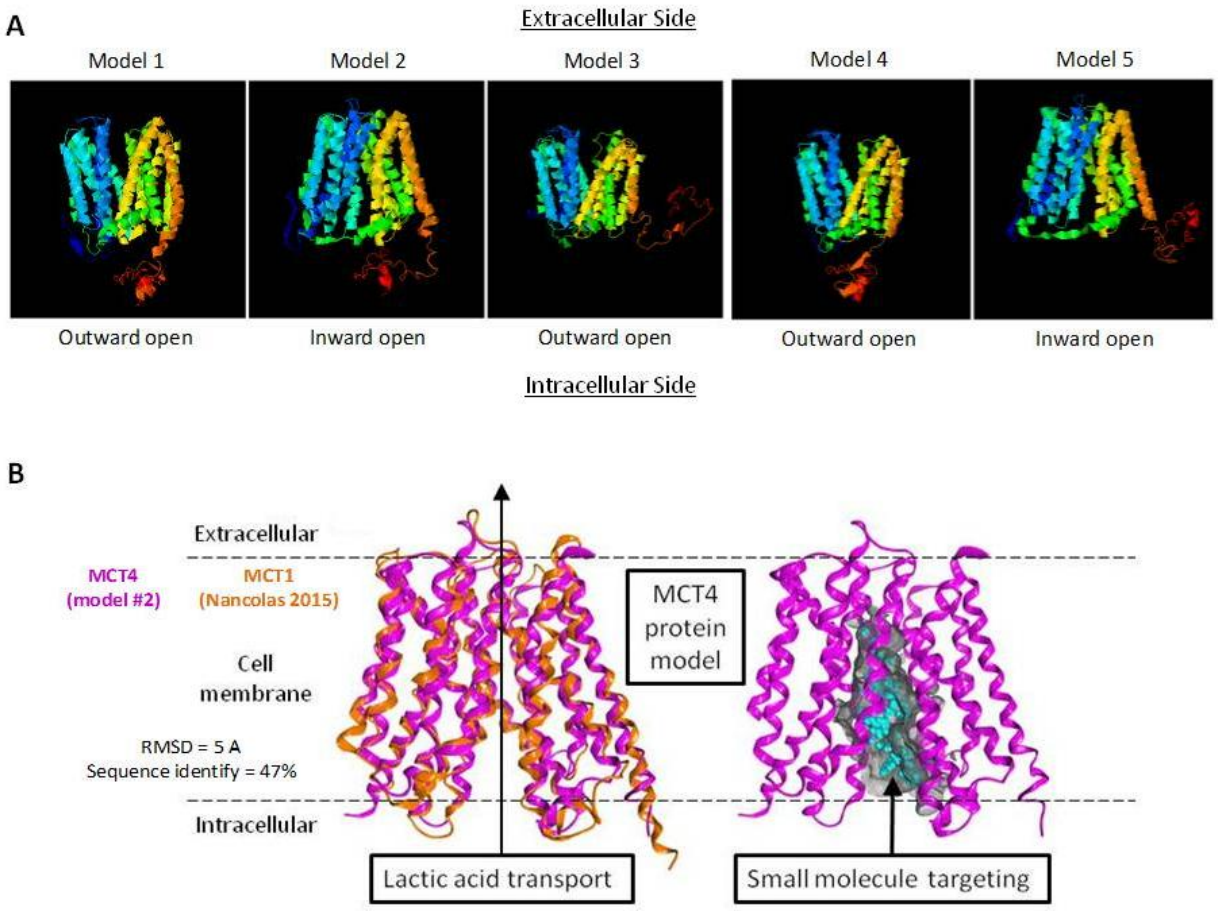

Figure 4.1. Modeling of the human MCT4 protein reveals a potential drug-binding pocket within the central channel towards the intracellular side. Multiple predicted structures for the human MCT4 protein were generated based on crystal structures of bacterial metabolite transporters using one of the best computational protein threading programs, I-TASSER (A). One such model, MCT4 model #2 (purple ribbon), resembled the inward-open conformation of a previously published MCT1 structural model (orange ribbon). Structural superimposition resulted in a moderate RMSD of 5Å due to variations in protein sequence. The MOE drug design program and virtual atomic probes (cyan spheres) have identified a suitable drug-binding pocket located at the center of the MCT4 protein model (B). Small molecule binding at this pocket has the potential to block MCT4 and prevent lactic acid transport.

drug-binding pocket may reside along the central canal. As such, chemical compounds that could bind with high affinity to the human MCT4 protein from within the channel could potentially function as an inhibitor via steric hindrance of metabolite transport activity.

4.3.2 Initial Efficacy Screen Identifies Three Hit Compounds for Further Optimization

Virtual drug screening by docking 4 million drug-like compounds from the ZINC database into the MCT4 intra-channel drug-binding pocket identified 57 potential MCT4 SMIs with high rankings from all three molecular docking programs. These compounds were designated the VPC25000-series and purchased for experimental efficacy assessment.

A combination of factors and measurements were used to evaluate in a preliminary manner the efficacy and specificity of these compounds as MCT4 inhibitors. Proliferation of the U-251 MG human glioblastoma cell line served as the primary screen. This is because a significant reduction in cell proliferation can be observed following MCT4 but not MCT1 siRNA transfection, suggesting that these cells are sensitive to MCT4-specific targeting. Furthermore, a reduction in media acidification and a change in cell morphology from cobblestone-like to spindle-like can also be observed in U-251 MG cells following MCT4 siRNA treatment, thereby offering additional assessment criteria to rule out off-target effects. Alternatively, proliferation of the U-2 OS human osteosarcoma cell line can be inhibited by MCT1 but not MCT4 siRNA transfection. It was therefore used as a counterscreen to assess MCT4 specificity and identify compounds that may have undesired inhibitory effects on MCT1. Finally, proliferations of the A-498 and 786-O human renal carcinoma cell lines were unaffected by either MCT1 or MCT4 knockdown, and thus served as controls to identify potentially toxic chemicals. Taken together, compounds that can effectively inhibit U-251 MG cell proliferation, changing cell morphology and reducing media acidification, without affecting U-2 OS, A-498, or 786-O cell growth were considered potential hits for downstream *in silico* optimization.

All 57 compounds (VPC-25001 to VPC-25057) were initially assessed at a 50uM concentration for potential therapeutic effects. Three compounds (VPC-25009, VPC-25013, and VPC-25041) appeared

to be particularly active, and 17 others seemed to be possible additional hits (Table 4.1). Treatment with compound #9, #13, and #41 were able to reduce U-251 MG cell proliferation (Figure 4.2A) with an accompanying change in cell morphology (Figure 4.2B). More detailed dose dependence analyses of these compounds suggest that they have IC_{50} values in the micromolar range (Figure 4.2C).

Validation of the treatment efficacy of VPC-25009, VPC-25013, and VPC-25041 in advanced PCa cell lines revealed that they are indeed therapeutically effective (Figure 4.2D). Furthermore, as expected of potential MCT4 SMIs and in keeping with earlier observations in our proof-of-concept study using MCT4 ASOs, these initial hit compounds were able to inhibit lactic acid secretion and glucose consumption (Figure 4.2E). Strikingly, these results are in contrast to the MCT1/2-specific inhibitor developed by AstraZeneca (AZD3965) currently in Phase I clinical trials. Our efficacy screens demonstrated no therapeutic efficacy for AZD3965 even at concentrations over 5,000-fold higher than the published IC_{50} of 1.6nM [414]. As such, it appears that our *in silico* drug development strategy may be able to successfully yield potent MCT4 inhibitors that are even more efficacious than those currently in clinical development.

4.3.3 First-round Optimization Suggests VPC-25009 Analogues are Potential MCT4 SMIs

The three hit compounds from the initial screen VPC-25009, VPC-25013, and VPC-25041 were further optimized *in silico* by assessing the goodness-of-fit of their related, purchasable chemical analogues into the proposed MCT4 drug-binding pocket. An additional 43 compounds (VPC-25058 to VPC-25100) sharing similar chemical scaffolds to compound #9, #13, and #41 were identified to have optimal molecular docking scores. These first-round optimized compounds were purchased and evaluated experimentally following the same methodology as the initial screen.

Screening results using a reduced dose of 25uM indicate that these first-round optimized analogues, if effective, show broad characteristics distinguishable based on the parental compounds (Table 4.2). More specifically, VPC-25041 analogues that show potential therapeutic efficacy appear to be generally toxic, inhibiting cell proliferation in all four assessed cell lines. On the other hand, VPC-

Table 4.1. Summary of results from the initial efficacy screen of VPC-25001 to VPC-25057. Percent inhibitions of cell proliferation following 50uM treatment of VPC-25001 to VPC-25057 are indicated by progressive shades of green (no inhibition) to red (extensive inhibition). Based on these results, VPC-25009, VPC-25013, and VPC-25041 can be considered initial hits. Seventeen other compounds show borderline efficacy and could be possible additional hits.

	MTS (144hrs)				Cell Count (72hrs)		Effective (>30%)
	MCT1/4 Insensitive		MCT1 Sensitive		MCT4 Sensitive		
	A-498	786-O	U-2 OS		U-251 MG		
	#1	#1	#1	#2	#1	#2	
Ctrl siRNA	0.00	0.00	0.00	0.00	0.00	0.00	
MCT1 siRNA	-16.61	-15.70	84.38	82.81	20.57	30.47	Yes (U-2OS)
MCT4 siRNA	-11.61	-19.11	43.82	36.36	34.83	42.32	Yes (U251MG)
DMSO	0.00	0.00	-21.19	1.55			
AZD3965			-2.35	23.14	-5.55	15.37	No
VPC-25001	4.81	1.17	-13.56	14.05	7.23	3.07	No
VPC-25002	12.86	5.24	-2.25	8.45	5.11	24.59	Maybe
VPC-25003	21.14	18.94	-25.97	14.05	13.38	27.25	No
VPC-25004	12.06	3.74	-3.95	13.15	11.31	30.94	Maybe
VPC-25005	12.54	2.44	-0.72	15.89	-8.28	19.88	No
VPC-25006	9.22	2.53	6.15	14.46	-17.70	6.56	No
VPC-25007	15.31	0.39	6.81	13.44	11.03	19.15	Maybe
VPC-25008	3.87	-0.64	14.14	14.51	5.98	-10.95	No
VPC-25009			37.15	74.33	34.64	-4.23	Yes
VPC-25010	1.82	-1.68	-7.60	15.27	-31.11	-9.45	No
VPC-25011	7.01	4.94	39.58	19.59	3.46	18.91	Maybe
VPC-25012	28.55	0.36	-7.04	14.60		25.57	No
VPC-25013	10.98	3.55	-9.69	17.83	46.01	43.86	Yes
VPC-25014	4.57	-1.88	-17.45	9.43		7.71	No
VPC-25015	5.89	1.27	-15.87	7.42	-3.43		No
VPC-25016	2.49	3.93	-2.89	12.64	-4.41		No
VPC-25017	1.92	1.14	-9.87	1.06	-7.37		No
VPC-25018	1.71	0.60	-9.50	2.95	-29.46		No
VPC-25019	2.31	1.00	-4.88	7.80	-7.93		No
VPC-25020	1.26	1.89	-12.20	5.77	1.42		No
VPC-25021	2.55	0.86	-9.95	1.40	10.66		No
VPC-25022	1.32	0.61	-7.55	11.01	19.04		No

Table 4.1. (Continued)

	MTS (144hrs)				Cell Count (72hrs)		Effective (>30%)
	A-498	786-O	U-2 OS		U-251 MG		
	MCT1/4 Insensitive		MCT1 Sensitive		MCT4 Sensitive		
	#1	#1	#1	#2	#1	#2	
VPC-25023	3.25	-0.31	-15.43	18.21	33.78		Maybe
VPC-25024	12.15	3.98	-19.88	12.76	35.03		Maybe
VPC-25025	0.83	1.64	-17.19	20.21	1.52		No
VPC-25026	0.39	0.68	-24.35	16.71	2.28		No
VPC-25027	1.58	1.06	-23.44	75.83	-3.05		Maybe
VPC-25028	-0.04	0.57	-17.99	20.30	24.16		No
VPC-25029	2.80	2.16	-14.66	24.54	22.34		No
VPC-25030	0.35	0.12	-18.25	47.87	6.31		Maybe
VPC-25031	1.16	-0.25	-19.77	21.65	-2.10		No
VPC-25032	15.49	1.54	-21.47	22.01	35.80		No
VPC-25033			-21.95	38.71	47.84		Maybe
VPC-25034	0.31	-1.68	-23.68	-27.50	-21.30		No
VPC-25035	2.19	0.83	-12.48	-22.03	-15.43		No
VPC-25036	1.23	1.12	-6.12	-16.75	-10.49		No
VPC-25037	11.42	5.32	-6.80	-12.91	41.20		Maybe
VPC-25038	34.49	3.57	-13.06	-2.51	52.90		No
VPC-25039	3.45	1.83	-19.19	-18.20	30.00		Maybe
VPC-25040	-0.52	0.64	-10.88	-23.34	-21.91		No
VPC-25041	92.45	76.94	86.86	67.44	32.19		Yes
VPC-25042	11.91	1.57	-30.78	-19.04	-2.63		No
VPC-25043	-0.90	1.36	-10.42	-25.53	-25.29		No
VPC-25044	-9.14	-9.72	17.77	53.42	3.34		Maybe
VPC-25045	72.81	38.52	42.56	52.08	19.59		Maybe
VPC-25046	-1.52	-1.01	-34.65	10.78			Maybe
VPC-25047	91.52	91.96	53.76	83.99	100.00		Toxic
VPC-25048	85.00	73.34	82.82	80.20	93.54		Toxic
VPC-25049	-0.29	-0.76	-48.62	-26.28	-42.03		No
VPC-25050	94.40	95.04	37.19	42.42	96.59		Toxic
VPC-25051	-0.56	-0.66	-47.10	-19.87	-49.20		No
VPC-25052	40.03	4.60	58.59	-2.09	77.16		Toxic
VPC-25053	0.89	0.89	-35.92	-11.69	-40.32		No
VPC-25054	-2.87	-0.33	29.26	64.83	47.91		Maybe
VPC-25055	2.14	0.37	-42.18	-22.80	31.84		Maybe
VPC-25056	-1.40	0.96	-32.22	-11.21	29.88		Maybe
VPC-25057	-2.79	-2.11	-32.49	1.89	0.78		No

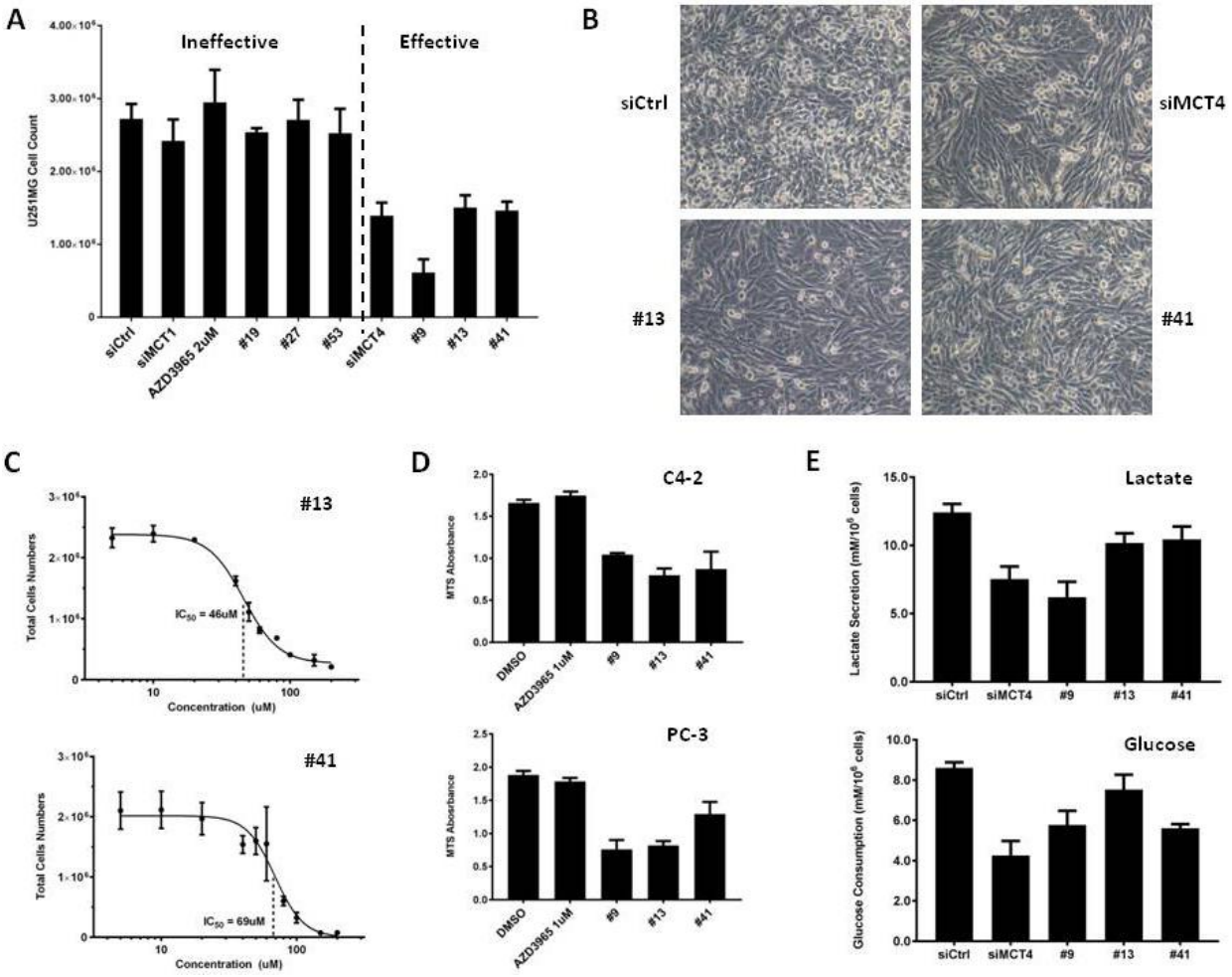

Figure 4.2. Representative data from the initial efficacy screen showing VPC-25009, VPC-25013, and VPC-25041 as potential hit compounds. Virtual MCT4 drug screening yielded 57 compounds for experimental assessment, some of which have efficacy comparable to MCT4 siRNA treatment as measured by cell proliferation (A). Initial effective hits were also able to induce a morphology change in U-251 MG cells from being cobblestone-like to becoming spindle-like, similar to MCT4 siRNA (B). More detailed assessment of VPC-25009, VPC-25013, and VPC-25041 indicate that these compounds have micromolar IC_{50} values (C) and demonstrate initial efficacy inhibiting advanced PCa cell proliferation (D). Treatment with compound #9, #13, and #41 also resulted in a reduction in lactic acid secretion and glucose consumption as measured by the respective colorimetric assays (E). These results are in agreement with previous observations from our proof-of-concept study inhibiting MCT4 function using MCT4 ASOs.

Table 4.2. Summary of results from the first-round optimization of VPC-25009, VPC-25013, and VPC-25041. Percent inhibitions of cell proliferation following 25uM treatment of the chemical analogues VPC-25058 to VPC-25100 are indicated by progressive shades of green (no inhibition) to red (extensive inhibition). Based on these results, VPC-25009 analogues appear to be the most promising, with a majority of them being either effective or potentially effective. Conversely, VPC-25013 analogues appear to have minimal efficacy while VPC-25041 are broadly toxic.

		MTS (144hrs)				Cell Count (72hrs)			Effective (>30%)
		A-498	786-O	U-2 OS		U-251 MG			
		MCT1/4 Insensitive		MCT1 Sensitive		MCT4 Sensitive			
Analogue	At 50uM	#1	#1	#1	#2	#1 25uM	#2 10uM	#3 5uM	
VPC #9	VPC-25081	34.28	51.37	21.68	24.09	16.33			Maybe
	VPC-25082	3.54	-22.88	4.77	7.95	19.12			Maybe
	VPC-25083	4.73	-24.88	-0.25	3.67	18.78			Maybe
	VPC-25084	3.38	-18.50	2.87	7.98	6.25			No
	VPC-25085			22.35	26.83	53.06	9.11	-21.38	Yes
	VPC-25086	49.41	84.24	10.26	34.67	16.77			Maybe
	VPC-25087	2.20	1.30	1.56	26.35	34.73	30.44	10.34	Yes
	VPC-25088	1.02	-33.52	1.80	6.20	17.08			Maybe
	VPC-25089	1.77	-58.47	21.59	20.91	39.12	10.84	-1.15	Yes
	VPC-25090	25.14	80.92	72.15	70.29	19.19			Maybe
	VPC-25091	30.19	79.19	27.95	12.71	-3.47			No
	VPC-25092			1.01	2.54	36.97	-10.84	-10.11	Yes
	VPC-25093	0.47	-46.79	1.70	9.50	-7.92			No
	VPC-25094	6.02	38.75	54.96	53.40	55.81	33.89	13.79	Yes
VPC #41	VPC-25058	93.71	94.14	92.31		100.00		13.46	Toxic
	VPC-25059	94.81	95.01	91.47		100.00		4.41	Toxic
	VPC-25060	94.88	95.35	92.32		100.00		19.95	Toxic
	VPC-25073	-1.52	0.77	-5.05		-14.29			No
	VPC-25074	0.83	-0.63	-11.03		-20.90			No
	VPC-25078	18.01	84.44	10.09		-46.33			No
	VPC-25079	2.57	4.50	1.00	15.79	-55.77			No
	VPC-25097	94.97	88.93	73.81		65.85		0.00	Toxic
	VPC-25098	93.34	85.49	73.31		76.12	26.53	22.20	Toxic
	VPC-25099	95.20	88.47	70.66		79.62	2.96	16.94	Toxic
	VPC-25100	94.87	88.34	74.77		100.00	34.14	18.56	Toxic

Table 4.2. (Continued)

		MTS (144hrs)				Cell Count (72hrs)			Effective (>30%)
		A498	786-O	U-2OS		U251MG			
		MCT1/4 Insensitive		MCT1 Sensitive		MCT4 Sensitive			
Analogue	**At 50uM**	#1	#1	#1	#2	#1 25uM	#2 10uM	#3 5uM	
VPC #13	VPC-25061	-3.01	-0.58	-1.71		7.45			No
	VPC-25062	-3.31	1.84	3.14		4.75			No
	VPC-25063	2.30	0.24	-0.71		-0.26			No
	VPC-25064	0.52	-0.55	4.19		14.95			Maybe
	VPC-25065	1.60	-0.02	32.94		-20.04			No
	VPC-25066	1.92	0.57	16.84		-23.12			No
	VPC-25067	1.82	-1.68	19.30		-49.45			No
	VPC-25068	1.67	0.70	11.98		-35.09			No
	VPC-25069	-0.59	0.09	-0.54		-45.01			No
	VPC-25070	-2.99	1.19	1.20		2.53			No
	VPC-25071	21.81	2.42	87.84		36.23	-7.64	0.00	Yes
	VPC-25072	2.40	1.53	-3.76		8.23			No
	VPC-25075	0.70	0.18	0.02		-11.38			No
	VPC-25076	-0.78	0.40	6.03		30.47	8.13	15.17	Yes
	VPC-25077	-1.26	0.56	5.25		-40.93			No
	VPC-25080	2.10	-16.11	-1.70	-3.09	-35.20			No
	VPC-25095	0.06	2.47	-4.27	3.38	-0.69			No
	VPC-25096	-0.31	5.21	2.43	15.31	16.29			Maybe

25013 analogues appear to have more subdued efficacy, with only 2 of 18 compounds (11%) showing potential therapeutic effects. Finally, five of 14 compounds (36%) chemically similar to VPC-25009 can be considered effective with six others also being potential hits, suggesting that the chemical scaffold underpinning VPC-25009 analogues are more robust and amenable to continued iterative optimization.

The observation that VPC-25009 and its chemical analogues may yield potentially potent and specific MCT4 SMIs is further confirmed by a more detailed characterization of the potentially effective analogues. The two effective VPC-25013 analogues VPC-25071 and VPC-25076 have IC_{50} values of 36uM and 15uM respectively, compared to the parental IC_{50} of 46uM (Figure 4.3A). This suggests that the optimization process can indeed yield compounds with improved therapeutic efficacy. More importantly, effective VPC-25009 analogues have even lower IC_{50} values, mostly around 10uM (Figure 4.3B). This lends additional evidence that the chemical scaffold surrounding VPC-25009 and its analogues may be more potent. Further assessment of treatment effects on lactic acid secretion and glucose consumption suggests that the effective first-round optimized analogues can also inhibit glucose metabolism via aerobic glycolysis in U-251 MG cells (Figure 4.3C). Taken together, further optimization and characterization of VPC-25009 and its related, effective chemical analogues, either through additional *in silico* approaches or through more traditional medicinal chemistry methodologies, could lead to the identification of potent and specific MCT4 SMIs. Improved versions of the current initial hits could thus be used for future preclinical efficacy and toxicology studies, ultimately yielding a drug candidate applicable for the clinical assessment of MCT4 inhibition as a therapeutic strategy for treatment of advanced PCa.

4.4 Discussion

Here we used an *in silico* drug discovery pipeline established at the VPC to make significant progress towards the development of potent and specific MCT4 SMIs for therapeutic applications treating advanced PCa. Employing state-of-the-art computational methodologies, we were able to build a structural model of the human MCT4 protein and virtually screen four million compounds, identifying 57

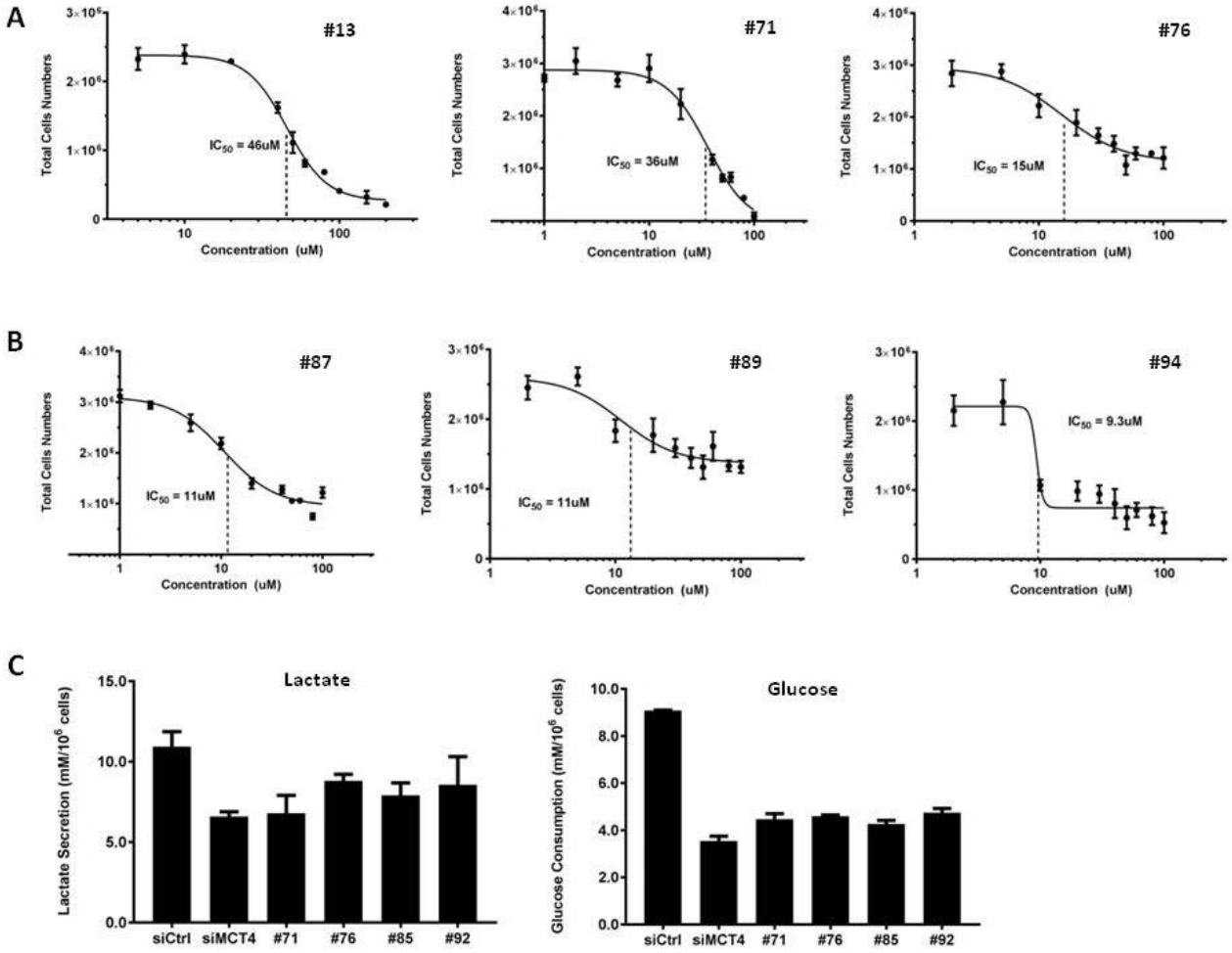

Figure 4.3. Representative data from the first-round optimization shows improved efficacies from a number of chemical analogues. First-round optimization yielded 43 chemical analogues of VPC-25009, VPC-25013, and VPC-25041 for experimental assessment. A measurement of cell proliferation indicates that the effective analogues of VPC-25013 show improved efficacy with lower IC_{50} values compared to the parental compound (A). Furthermore, effective analogues of VPC-25009 have even greater potencies at inhibiting cell proliferation with IC_{50} values around 10uM (B). Importantly, colorimetric measurements of lactic acid and glucose levels show that the effective chemical analogues are also able to inhibit lactic acid secretion and glucose consumption as expected of MCT4 inhibition (C).

predicted hits for initial experimental evaluations. The MCT4 model closely resembles the published structural model of human MCT1 with an inward-open resting conformation. A potential drug-binding pocket was also identified within the central channel similar to the proposed binding site in MCT1 for the MCT1/2 specific inhibitor AZD3965 [532, 540].

Virtual drug screening at the identified drug-binding site yielded an initial list of 57 purchasable compounds with high rankings in all three molecular docking programs. Experimental assessment of their efficacies suggests that VPC-25009, VPC-25013, and VPC-25041 may be particularly effective and relatively specific inhibitors of MCT4, with 17 other compounds being additional potential hits. More specifically, the three initial hits were able to reduce the proliferation of MCT4 inhibition-sensitive and advanced PCa cells. Furthermore, treatment with compounds #9, #13, and #41 also inhibited lactic acid secretion and glucose consumption similar to earlier *in vitro* observations following MCT4 knockdown. A first-round *in silico* optimization assessing the chemical analogues of VPC-25009, VPC-25013, and VPC-25041 yielded 43 additional compounds for experimental evaluation. These analogues appear to have general properties distinguishable based on the parental compound. For example, VPC-25041 analogues appear to be broadly toxic, inhibiting cell proliferation across all four assessed cell lines. In contrast, VPC-25013 analogues appear to have more subdued efficacy, with only two compounds being potentially effective. Importantly, VPC-25009 analogues appear to be the most promising hit series, with the majority of compounds remaining effective or somewhat effective. As such, the chemical scaffold underlying VPC-25009 may fit best into the proposed binding pocket and be more amenable for further optimizations into potent and specific MCT4 inhibitors.

These preliminary results indicate that a computer-assisted drug development strategy can indeed be effective at replacing randomized high-throughput screens, especially in the early stages of hit discovery. Given that traditional unbiased screening approaches generate on average 10 lead compounds per target [541], our ability to identify 3 hit compounds with 17 additional possibilities by molecular docking suggests that this computational approach is highly beneficial, particularly in light of an accelerated hit discovery process with drastically reduced financial, material, and labour costs.

Furthermore, our successful hit identification and first-round optimization of potential MCT4 SMIs lend support to the fact that an *in silico* strategy can be applied even to more difficult drug targets without available crystal structures – in our case a membrane channel protein with minimal extra-membrane domains.

Finally, the VPC-25009 analogues appear to be a promising hit series. Our experimental results suggest that these compounds share a potent core scaffold exhibiting potential MCT4-inhibitory properties. Analysis for the second-round *in silico* optimization of this series using more detailed modeling approaches suggests that the most effective analogues (VPC-25087 in particular) retain important hydrophobic interactions with other transmembrane domains in the central channel. More importantly, these compounds are also able to maintain strong interactions with arginine-278 in the drug-binding pocket, a key residue involved in facilitating lactate-transport analogous to arginine-306 in mouse MCT1 [532, 542]. The convergence of our modeling approach to known MCT1 biology and the iterative improvements in therapeutic efficacy and predicted binding potentials through multiple rounds of screening lend increasing confidence that our *in silico* platform can accurately reflect MCT4 protein structure and function. Ongoing efforts are underway to experimentally demonstrate direct binding between our inhibitors and the human MCT4 protein. More specifically, site-directed mutagenesis of key residues surrounding the proposed binding site could verify our binding hypothesis, and confirmation of MCT4-specificity without inhibition of other MCT family members would lend further support for our conclusions here.

Taken together, using a computer-aided drug discovery pipeline to identify potential MCT4 SMIs has proved promising with significant progress towards generating and developing a candidate hit series for clinical use. While the full drug development process from hit generation to lead optimization to clinical candidate selection [402] is beyond the scope of this dissertation, further optimization of VPC-25009 analogues through additional *in silico* modeling, medicinal chemistry, and QSAR analysis could result in a lead compound with even higher MCT4 specificity and anticancer efficacy for eventual preclinical studies. A safe and efficacious lead compound could then be evaluated in the clinic, inhibiting

MCT4 function as a therapeutic strategy for treatment of advanced PCa patients. More significantly, as MCT4-mediated excessive lactic acid secretion may be a widely common phenotype across multiple cancer types, the therapeutic use of an MCT4 SMI can have an even broader impact for the more generalized treatment of cancer.

Chapter 5: Summary and Conclusions

5.1 Summary

This doctoral study integrates a novel conceptual theory with state-of-the-art experimental approaches to advance the field of PCa biology and therapeutics. Effective treatment options for advanced PCa remain a significant unmet clinical need. While traditional approaches revolve around the inhibition of AR signaling, treatment resistance frequently develops. The problem is exacerbated by the current use of next-generation antiandrogens, promoting an increasing number of recurrences in a truly androgen-independent manner. One such mechanism of castration-resistance is transdifferentiation into NEPC, a highly aggressive subtype of PCa currently with no effective therapeutic options. As such, the development of a treatment approach independent of AR signaling is becoming increasingly necessary for both traditional CRPC and emergent NEPC.

Detailed in Chapter 1, we proposed a novel theory for understanding altered cancer metabolism, a known hallmark cancer characteristic. Altered utilization of metabolites is most commonly observed as elevated aerobic glycolysis in cancers. The current consensus of scientific thought suggests that this metabolic phenotype allows cancer cells to retain sufficient molecular building blocks for biomacromolecule synthesis during proliferation, with excessive lactic acid production and secretion relegated as a process of waste management. However, we suggest that the excessive production and secretion of lactic acid can actually be a mechanism by which cancer cells actively stimulate multiple tumour-promoting processes. There is growing evidence in the literature that an acidic tumour microenvironment as created by cancer-generated lactic acid can facilitate angiogenesis, local tissue invasion, epigenetic alterations, and resistance to hypoxic stress. More importantly, the role of an acidic tumour microenvironment in suppressing the local anticancer immune response has thus far been largely overlooked. As such, we integrated multiple lines of clinical and experimental observations and synthesized a novel hypothesis describing the central tumour-promoting and immunosuppressive role of cancer-generated lactic acid. Taken together, the inhibition of excessive lactic acid secretion from cancer cells could be a promising therapeutic strategy, reversing multiple lactate-associated downstream

processes fundamental to cancer growth and survival. More specifically, the inhibition of MCT4-mediated lactic acid secretion could suppress cancer cell proliferation and enhance anticancer immunity. Together with potentially reducing angiogenesis, local tissue invasion, and distant metastasis, a therapeutic strategy inhibiting MCT4 function could thereby synergistically enhance treatment efficacy, achieving combinatorial effects with only a single-target agent. Furthermore, this novel hypothesis is broadly applicable across multiple cancer types. This suggests that in addition to advanced PCa, an effective treatment option arising from this theory could have therapeutic benefits for the clinical management of a wide range of cancer types.

We first set out to verify the involvement of elevated glycolysis and lactic acid production in advanced PCa tumours. As detailed in Chapter 2, we developed a metabolic pathway score to better utilize existing transcriptomic information available for our panel of PCa PDX models and public PCa patient datasets. An initial analysis of treatment-naive primary PCa samples suggests that our novel methodology can indeed accurately reflect tumour metabolic phenotypes. Broad heterogeneity was observed across multiple metabolic pathways, with only a portion of primary PCa PDX and patient tumours showing signatures of elevated glycolysis and increased lactic acid production. However, when the analysis was applied to advanced PCa samples of CRPC and NEPC, a striking increase in reliance on aerobic glycolysis can be observed. Significantly, increased glycolysis and lactic acid production as mediated by MCT4 overexpression seems to be a defining metabolic phenotype of NEPC PDX and patient tumours. To our knowledge, we present here the first description of NEPC metabolism suggesting that elevated aerobic glycolysis is indeed a clinically relevant characteristic for this aggressive PCa subtype. Furthermore, as elevated glycolysis and excessive MCT4-mediated lactic acid secretion is associated with advanced PCa including both CRPC and NEPC, a therapeutic strategy inhibiting MCT4 function could prove effective.

Following our observations confirming elevated aerobic glycolysis as a metabolic phenotype clinically relevant to advanced PCa, we set out to experimentally demonstrate its functional importance by inhibiting MCT4 in a proof-of-concept study. As detailed in Chapter 3, a panel of human MCT4-

specific ASOs were designed and verified as effective at decreasing MCT4 expression. Transfection of the most promising ASOs into multiple advanced PCa cell lines *in vitro* resulted in significant inhibition of cell proliferation. Reduction of MCT4 expression also resulted in decreased invasion and migration abilities. A more detailed assessment of alterations to glucose metabolism following MCT4 knockdown revealed that lactic acid secretion and glucose consumption was inhibited in both CRPC and NEPC cell lines. Strikingly, a common downregulation of multiple upstream enzymes and regulators of glycolysis and lactic acid production was observed in both cell types after MCT4 ASO transfection, suggesting that an overall inhibition of glucose metabolism is an important mechanism of action. Our findings were confirmed *in vivo*, where MCT4 ASO treatment reduced PC-3 tumour growth. More importantly, however, our use of *in vivo* models with residual functional immune cells demonstrated that MCT4 inhibition can indeed enhance anticancer immunity. MCT4 ASO treatment increased activated NK cell proportions in PC-3 tumour bearing nude mice and stimulated proliferation and differentiation of patient $CD8^+$ cytotoxic T lymphocytes in a novel first-generation PCa PDX model. Taken together, our proof-of-concept study confirmed the functional role of MCT4-mediated lactic acid secretion in facilitating the cancer hallmarks of sustained proliferation, altered metabolism, invasion/metastasis, and avoiding immune destruction. Furthermore, our results suggest that MCT4 ASOs could indeed be a promising therapeutic agent, with next-generation base modifications potentially increasing the clinical applicability of our patented MCT4 ASO sequences.

Given the strong preliminary evidence that MCT4 function is indeed critical to multiple tumour-promoting processes including assisting cell proliferation and suppressing local anticancer immunity, we embarked on developing, in parallel to MCT4 ASOs, a potential MCT4 SMI for clinical use. The initial steps of this developmental process are described in Chapter 4. By using a state-of-the-art *in silico* drug discovery pipeline established at the VPC, we were able to construct a structural model of human MCT4 with a potential drug-binding pocket within the central channel. Virtual screening of four million purchasable drug-like compounds extracted from the ZINC database identified 57 potential hits for initial experimental evaluation. Three compounds, VPC-25009, VPC-25013, and VPC-25041 appear to be

particularly effective and were carried forward into first-round *in silico* optimization assessing their related chemical analogues. Experimental evaluations of the resultant 43 optimized compounds revealed that VPC-25041 analogues are broadly toxic while VPC-25013 analogues are only modestly effective. In contrast, the majority of VPC-25009 analogues retained or improved initial efficacy, suggesting the presence of a potent underlying chemical scaffold. As such, VPC-25009 analogues are a promising hit series, and further optimization and downstream evaluations could yield potent and specific MCT4 SMI candidates for clinical use.

Taken together, this doctoral study proposed a novel hypothesis describing the central tumour-promoting and immunosuppressive role of cancer-generated lactic acid and provided initial experimental evidence in preliminary support. More importantly, this new paradigm of altered cancer metabolism could result in an effective therapeutic strategy inhibiting multiple downstream cancer characteristics for treatment of advanced PCa and beyond. Two potential therapeutic modalities for inhibiting MCT4 function were explored. The results suggest that such a treatment strategy could be efficacious, and with additional experimental investigations and validations, could be clinically applicable for the treatment of multiple advanced malignancies.

5.2 Ongoing Work and Future Directions

Ongoing work and future directions for this project can largely be considered in terms of the strengths and limitations of the current study. Regarding our assessment of metabolic phenotypes in advanced PCa as detailed in Chapter 2, we recognize that gene expression data is only an indirect estimation of metabolic profiles. While analysis using our novel metabolic pathway scores is sufficient for the purposes of emphasizing better utilization of existing data and providing rationale for downstream biological evaluations, more direct metabolic measurements would offer stronger evidence in support of our claims. For example, assessment of the various advanced PCa PDX models using FDG-PET could provide a quick confirmation whether elevated glycolysis and increased glucose consumption is a true biological phenomenon, particularly in our NEPC PDXs. Alternatively, stable isotope tracing experiments

[470, 471] could be performed with the PDX models as well, using mass spectrometry to determine the metabolic fate of ^{13}C-glucose and whether disproportionate amounts of lactic acid maybe produced and potentially secreted into the tumour microenvironment. Finally, a metabolomics approach could also be used to assess both PDX and patient advanced PCa tumours. While we focused on changes to glucose metabolism in our present study, a more global characterization of metabolic activity could potentially reveal altered utilization of other metabolic pathways. A more comprehensive description of CRPC and NEPC metabolism integrating both transcriptomic and metabolomic data could thus identify additional therapeutic avenues associated with pathological changes to tumour energetics in advanced PCa.

Regarding inhibition of MCT4 function by knockdown using ASOs as described in Chapter 3, ongoing work is being done to evaluate next-generation nucleotide chemistry for clinical application purposes. As the majority of ASOs entering clinical trials employ generation 2.5 bicyclic cEt modifications, we are currently investigating whether improved ASOs yield better efficacy and knockdown efficiency. If the generation 2.5 MCT4 ASOs are truly more effective, *in vivo* studies using a panel of PDX models can provide additional preclinical data regarding treatment efficacy. PDX tumours offer a number of advantages over traditional cell line tumours as employed in the current study. Not only are PDX models more reflective of clinical patient tumours from a histological and biological perspective, the assessment of treatment efficacy using multiple PDX tumour lines can also better mimic potential outcomes in a diverse patient population as encountered in the clinic. Our available panel of six CRPC and five NEPC PDXs can thus help overcome common limitations associated with the over-reliance on a single *in vivo* model to inform clinical trial direction. In particular, as the genetic and molecular features of these PDXs are already exhaustively documented, characteristics common to models that respond well to MCT4 inhibition could inform patient selection criteria and aid future clinical trial design. Separately, the immunostimulatory effects of MCT4 inhibition could be verified using additional first-generation PDX tumours as tissue availability allows. While the initial assessment reported here provides promising preliminary data in support of our hypothesis, we recognize that it is only representative of one patient tumour. As such, a similar stimulation of the anticancer immune response across multiple first-generation

PDXs following MCT4 inhibition would suggest that our hypothesis is indeed broadly descriptive of advanced PCa tumour biology. Finally, the therapeutic effects of MCT4 ASO treatment in other cancer types are also being investigated. Given the propensity of ASOs to accumulate in the liver and kidneys, expanding the application of MCT4 ASO treatment to liver and renal malignancies can potentially exploit its biodistribution characteristics and result in even greater therapeutic efficacy.

Finally, regarding the development of potent and specific MCT4 SMIs as mentioned in Chapter 4, second-round *in silico* optimization has identified 25 additional compounds sharing the common VPC-25009 chemical scaffold. These analogues have even better predicted binding to the proposed MCT4 drug-binding pocket than VPC-25087 and will be experimentally assessed for improved efficacy. While our initial results through iterative round of optimization provide increasing confidence that our modeling approach can accurately reflect MCT4 structure and function, the computational nature of our drug discovery pipeline still requires experimental verification. Given the fact that the MCT4 model employed is a structural prediction, further investigations are required to ascertain drug binding and isoform specificity. For example, direct binding assays using radiolabelled hit compounds can determine binding affinities to MCT4 in comparison to other MCT family members. Furthermore, site-directed mutagenesis of key residues in our proposed binding hypothesis can help validate the accuracy of our *in silico* modeling strategies. Finally, recent technological advances in cryo-EM can facilitate protein structural analysis at a level comparable to traditional X-ray crystallography techniques [521, 522]. Binding of hit compounds to MCT4 can thus be directly observed, potentially confirming our predicted MCT4 structural model or, alternatively, providing an accurate structure to revise our hit discovery process. Following confirmation of direct binding and MCT4 specificity, hit compounds can be iteratively improved by additional *in silico* optimizations, medicinal chemistry, and QSAR analysis to arrive at a potent and specific lead compound. Its therapeutic efficacy can then be assessed in a panel of advanced PCa PDX models similar to strategies proposed for MCT4 ASOs. Pharmacology and toxicology studies can also be done in preparation for the initiation of clinical trials. Uniquely, as our candidate MCT4 ASO sequences do not affect mouse MCT4 expression, a lead compound that also effectively inhibits mouse MCT4

function can help fully assess the immunomodulatory effects of MCT4 inhibition in immunocompetent models of murine PCa.

5.3 Conclusions and Significance

In conclusion, this doctoral dissertation expanded the current understanding of PCa biology in both a theoretical and experimental manner. Conceptually, we proposed and were able to confirm with initial experimental evidence that lactic acid is indeed important to multiple cancer hallmarks, linking the fundamental properties of sustained proliferation, angiogenesis, and invasion/metastasis with altered cellular energetics. More significantly, our hypothesis elevates the relatively underappreciated ability of an acidic tumour microenvironment as induced by cancer-generated lactic acid to facilitate local suppression of the anticancer immune response. If proven true, a therapeutic approach targeting MCT4-mediated lactic acid secretion, whether through effective ASOs or potent and specific SMIs, can offer combinatorially synergistic therapeutic benefits, inhibiting multiple downstream lactate-associated tumour-promoting processes with only a single therapeutic agent.

Experimentally, we were able to employ a number of novel techniques towards confirming our hypothesis. For example, we developed a unique metabolic pathway score to describe the metabolic phenotype of various tumours, resulting in the first-in-field confirmation of elevated glycolysis and increased lactic acid production as a clinically relevant metabolic feature of NEPC PDX and patient tumours. Furthermore, our panel of patented MCT4 ASO sequences offer a potential therapeutic agent for the reduction of MCT4 expression and function, decreasing proliferation and inhibiting glucose metabolism in advanced PCa cells. We were also able to demonstrate that MCT4 inhibition can potentially stimulate anticancer immunity, uniquely employing first-generation PDX tumours to assess patient tumour-associated immune cells. Finally, the use of a state-of-the-art computer-assisted drug discovery pipeline for the development of potent and specific MCT4 SMIs resulted in the identification of a new and promising hit series based on the chemical scaffold of VPC-25009.

Taken together, the development of an effective therapeutic strategy based on our novel conceptual understanding of excessive cancer-generated lactic acid could broadly impact a large patient population. Given the current lack of effective treatment options for many highly glycolytic late-stage cancers including advanced PCa, a successful therapeutic strategy targeting MCT4 function could result in the suppression of multiple fundamental processes critical to cancer growth and survival, inhibiting cell proliferation, angiogenesis, invasion/metastasis, and enhancing the anticancer immune response.

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Appendix A. Full List of Genes Assessed in the Calculation of Metabolic Pathway Scores

Cholesterol
ACAT1
ACAT2
CYP51A1
EBP
FDFT1
FDPS
GGPS1
HMGCR
HMGCS1
HMGCS2
IDI1
LBR
LSS
MVD
MVK
PMVK
TM7SF2

CDP Choline
CHDH
CHPT1
PCYT1A
PLD1
PLD2
SLC44A1

Choline/Phosphocholine
CHKA
PEMT
GDPD1
PLA2G7
LYPLA2
LYPLA1
SLC44A4

Fatty Acid Activation
ACSBG1
ACSL1
ACSL3
ACSL4
ACSL5
ACSL6
SLC27A1
SLC27A2
SLC27A3
SLC27A4
SLC27A5
SLC27A6

Gluconeogenesis
ALDOB
ENO1
ENO2
ENO3
FBP1
G6PC3
GAPDH
GPI
LDHB
MDH1
MDH1B
MDH2
PC
PCK2
PFKFB2
PFKFB3
PFKFB4
PGAM5
PGK1
TPI1

Fatty Acid B Oxidation
ACAA1
ACAA2
ACADM
ACAD10
ACAD11
ACAD8
ACAD9
ACADL
ACADS
ACADSB
ACADVL
ACAT1
ACAT2
ACSBG1
ACSL1
ACSL3
ACSL4
ACSL5
ACSL6
ECHS1
EHHADH
HADH
HADHA
HADHB
HSD17B4
SLC27A1
SLC27A2
SLC27A3
SLC27A4
SLC27A5
SLC27A6
CPT1A
CPT1B
CPT1C
CPT2

Ketogenesis
ACAT1
ACAT2
BDH1
HMGCS2
HMGCL
ACAA2

Ketolysis
ACAT1
ACAT2
BDH1
BDH2
OXCT1
OXCT2

Glycolysis
ADPGK
ALDOA
ALDOB
ALDOC
ENO1
ENO2
ENO3
FBP1
GAPDH
GCK
GPI
HK1
HK2
HK3
LDHA
PFKFB2
PFKFB3
PFKFB4
PFKL
PFKM
PGAM1
PGAM5
PKM2
TPI1

Lipogenesis
ACACB
ACAT1
ACAT2
ACLY
ACSBG1
ACSL1
ACSL3
ACSL4
ACSL5
ACSL6
ACSS1
ACSS2
ACSS3
CYP51A1
CPT1A
DBI
EBP
FADS1
FADS2
FASN
FDFT1
FDPS
GGPS1
HMGCR
HMGCS1
HMGCS2
IDI1
INSIG1
INSIG2
LBR
LDLR
LSS
MGLL
MVD
MVK
PMVK
SCD5
SREBF1
SREBF2
TM7SF2

Pentose Phosphate Pathway
H6PD
PGD
RPE
RPIA
TALDO1
TKT
PGLS

Glutaminolysis
GLS
GLS2
GLUD1
GLUD2
GOT1
GOT2
IDH1
IDH2
IDH3A
IDH3B
IDH3G
OGDH
OGDHL

Oxpho Complex V
ATP5A1
ATP5B
ATP5C1
ATP5D
ATP5E
ATP5F1
ATP5G1
ATP5G2
ATP5G3
ATP5H
ATP5I
ATP5J
ATP5J2
ATP5L
ATP5O
ATP5S

Oxpho Complex IV
COX10
COX11
COX15
COX17
COX4I1
COX4I2
COX5A
COX5B
COX6A1
COX6B1
COX6B2
COX6C
COX7A1
COX7A2
COX7A2L
COX7B
COX7C
COX8A
COX18
COX19
COX16

Oxpho Complex III
CYB5A
CYB5B
UQCRC1
UQCRC2

Oxpho Complex II
SDHA
SDHB
SDHC
SDHD

Proline Synthesis
PYCR1
PYCR2
PYCRL
OAT
ALDH18A1

Oxpho Complex I
NDUFA1
NDUFA10
NDUFA11
NDUFA12
NDUFA13
NDUFA2
NDUFA3
NDUFA4
NDUFA4L2
NDUFA5
NDUFA6
NDUFA7
NDUFA8
NDUFA9
NDUFAB1
NDUFB1
NDUFB10
NDUFB11
NDUFB2
NDUFB3
NDUFB4
NDUFB5
NDUFB6
NDUFB7
NDUFB8
NDUFB9
NDUFC1
NDUFC2
NDUFS1
NDUFS2
NDUFS3
NDUFS4
NDUFS5
NDUFS6
NDUFS7
NDUFS8
NDUFV1
NDUFV2
NDUFV3

TCA Cycle
ACO1
ACO2
CS
DLAT
DLD
DLST
FH
IDH1
IDH2
IDH3A
IDH3B
IDH3G
MDH1
MDH1B
MDH2
OGDH
OGDHL
PC
PCK2
PDHA1
PDHB
SDHA
SDHB
SDHC
SDHD
SUCLA2
SUCLG1
SUCLG2

Pyruvate
LDHB
LDHD
PDHA1
PDHB
DLAT
DLD

Lactate Production	**Proline Degradation**
PDK1	PRODH
LDHA	ALDH4A1
PKM2	PEPD
SLC16A1	GLUL
SLC16A3	
BSG	

Appendix B. Full List of MCT4 ASO Sequences Assessed

MCT4 ASO	Sequence (5' to 3')	MCT4 ASO	Sequence (5' to 3')
Rat	CACAGCTCCTCCCATGGCCAGG	#20	CCACTCTGGAATGACACGGT
#1	TCCCATGGCCAGGAGGGTTG	#21	GTAGGAGAAGCCAGTGATGAC
#1v1	ATGGCCAGGAGGGTTG	#22	AGCATGGCCAGCAGGATGGA
#1v2	CATGGCCAGGAGGGTT	#23	GGCTGGAAGTTGAGTGCCAA
#1v3	CCATGGCCAGGAGGGT	#24	CATGCCGTAGGAGATGCCAA
#1v4	CCCATGGCCAGGAGGG	#25	GGCCACCGCCTCCATCAGCA
#1v5	TCCCATGGCCAGGAGG	#26	CCTGAGCCAGTCCAGTTTGT
#2	GACCTGTCCCGTAGAGCATG	#27	CTCAGGCTGTGGCTCTTTGG
#3	GTCCCGGAAGACGCTCAGGT	#28	CCCACCCACCCTCCCATTAA
#4	TTCCCAAGCCCCGCCACGAA	#29	GCTTCTGTACCTCCTCCCTG
#5	AATGCTCCACCTCCCGCAAG	#30	TGTCGCTGTAGCCGATCCC
#6	ACCTCCCCGTTTTTCTCAGG	#31	TTAAAGTCACGTTGTCTCG
#7	TGTGAACCACCTCCCCGTTT	#32	TAGCGGTTCAGCATGATGA
#8	TCTGTACCTCCTCCCTGTGC	#33	TTGCGGCTTGGCTTCACCG
#9	GAATGACACGGTTCCCACCC	#34	AGCACGGCCCAGCCCCAGCC
#10	GCCCACCCACCCTCCCATTA	#35	GAGCTCCTTGAAGAAGACACT
#11	AAGAGACCCCCCACAAGCAT	#36	CAGGATGGAGGAGATCCAGG
#12	AAGGACGCAGCCACCATGCC	#37	AGACCCCCCACAAGCATGAC
#13	TTGGCGTAGCTCACCACGAA	#38	GAAGTTGAGTGCCAAACCCAA
#14	AGATGCAGAAGACCACGAGG	#39	CCCGTTGGCCATGGGGCGCC
#15	CCCACCATGCCGTAGGAGAT	#40	GCCAGCCCGTTGGCCATGGG
#16	AGTCCACCCCCGAGTCTGCA	#41	AGGAAGACAGGGCTACCTGC
#17	CTTCACCGCAGATCCACTCT	#42	GCACACAGGAAGACAGGGCT
#18	AACACTCCACCCACACGCAG	#43	CAGGGCACACAGGAAGACAG
#19	CCAGCCACTCAGACACTTGT	#44	CAGCAGTTGAGCAGCAGGCC

Appendix C. Full List of qPCR Primers Used in This Study

Sequence Designation		Sequence (5' to 3')
SLC16A3 (MCT4)	Forward	ACCCACAAGTTCTCCAGTGC
	Reverse	AGCAAAATCAGGGAGGAGGT
SLC16A1 (MCT1)	Forward	ATGGTGGAGGTCCTATCAGC
	Reverse	CAATCATGGTCAGAGCTGGA
SLC16A7 (MCT2)	Forward	AGGTGATCTGGGGAACCAAAG
	Reverse	TTGGTGGCATTTCTGCTCCTC
BSG (CD147)	Forward	CTACGAGAAGCGCCGGAAG
	Reverse	GACGTGGAGCAGGGAGCG
SLC2A1 (GLUT1)	Forward	CCTGCAGTTTGGCTACAACAC
	Reverse	CAGGATGCTCTCCCCATAGC
GAPDH	Forward	CACCAGGGCTGCTTTTAACTC
	Reverse	GACAAGCTTCCCGTTCTCAG
PGK1	Forward	GTGTTCCGCATTCTGCAAGCC
	Reverse	TTGGGACAGCAGCCTTAATCC
PGAM1	Forward	GCTAATCCCAGTCGGTGCC
	Reverse	GTCCGGATCGCTCTCTTCTG
ENO1	Forward	CCTGCCCTGGTTAGCAAGAA
	Reverse	GGGACTGGCAGGATGACTTC
ENO2	Forward	ATGGTGAGTCATCGCTCAGG
	Reverse	AGGCAAGCAGAGGAATCACA
PDK1	Forward	TTGAATACAAGGAGAGCTTTGGGGT
	Reverse	AATCACACAGACGCCTAGCATTTT
PDHA1	Forward	CGCTATGGAATGGGAACGTCTG
	Reverse	TCGTGTACGGTAACTGACTCC
LDHA	Forward	GGAAAGGCTGGGAGTTCACC
	Reverse	CTGGGTGCAGAGTCTTCAGAG
LDHB	Forward	CCAGGATTCATCCCGTGTCAA
	Reverse	CCCGGGCATTGAGGATACAT
RPL13A	Forward	GGAGCCAGAAGACTGATTGG
	Reverse	CCTGTAACCCCTTGGTTGTG

Sequence Designation		Sequence (5’ to 3’)
TUBA1B	Forward	GAGGTTGGTGTGGATTCTGTT
	Reverse	AGCTGAAATTCTGGGAGCAT
HPRT1	Forward	GGTCAGGCAGTATAATCCAAAG
	Reverse	CGATGTCAATAGGACTCCAGATG
Mouse SLC16A3 (MCT4)	Forward	TGCTGGCTATGCTCTATGGC
	Reverse	ATAGGGCGACGCTTGTTGAA
Mouse RPL13A	Forward	TTGTGGCCAAGCAGGTACTTC
	Reverse	CTCTTGGTCTTGTGGGGCAG
Mouse TUBA1B	Forward	GCCTTCTAACCCGTTGCTATC
	Reverse	GTGGGTTCCAGGTCTACGAA
Mouse HPRT	Forward	AGAGCGTTGGGCTTACCTC
	Reverse	GCAAGTCTTTCAGTCCTGTCC